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## The Role of PAK6 in Breast Cancer Cells

Babteen, Nouf Abubakr Mohammed

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# **The Role of PAK6 in Breast Cancer Cells**

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Division of Cancer Studies  
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This thesis is submitted to fulfill the requirements for the  
degree of Doctor of Philosophy

2016



## **Declaration of Authorship**

I declare that the work presented in this thesis is the work of the author and others work is fully acknowledged where included.

Nouf Babteen

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## **Abstract**

Cell migration and invasion play a key role in the breast cancer progression and are considered hallmarks of cancer. Migration requires the reorganization of the actin cytoskeleton, a process known to be regulated by Rho family GTPases via interaction with downstream effector proteins. p21-activated kinase (PAKs) are a family of six serine/threonine protein kinases, which are activated by the RhoGTPases Rac1 and/or Cdc42. PAKs have been implicated in many cellular process including cytoskeleton organization, cell motility, cell cycle progression, cell migration and cell survival. Overexpression of PAK isoforms has been found in different human cancer tissues including breast. Therefore, PAKs may provide a therapeutic target for the prevention breast cancer progression and metastasis. Whilst much is known about the biology of PAK1 less is known about the more recently described family member PAK6. Although, there is some evidence to suggest PAK6 is overexpressed in cancer cell lines relatively little is known about the role of PAK6 in cancer progression. This study detected endogenous PAK6 expression in multiple breast cancer cell lines. Moreover, in MDA-MB-231 cells expression of activated PAK6 induced cell rounding, whilst PAK6 deficient cells exhibit an elongated phenotype. Interestingly, this study has also found that treatment with Panobinostat reduces PAK6 expression and phenocopies the cell morphology observed for PAK6 knockdown cells. Furthermore, through immunoprecipitation and mass spectrometry analysis, interactions between PAK6 and unconventional RhoGTPases in breast cancer cells were identified in addition to interactions between PAK6 and regulators of cellular contractility. Taken together our results suggest that PAK6 can influence cell morphology via manipulation of RhoGTPase signalling.

## Table of Contents

<b>DECLARATION OF AUTHORSHIP .....</b>	<b>2</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>3</b>
<b>ABSTRACT .....</b>	<b>5</b>
<b>LIST OF FIGURE .....</b>	<b>8</b>
<b>LIST OF ABBREVIATIONS .....</b>	<b>12</b>
1. CHAPTER 1: INTRODUCTION .....	16
1.1. <i>Breast cancer</i> .....	16
1.1.1. Breast Cancer Progression .....	17
1.2. <i>Carcinoma Metastasis</i> .....	19
1.3. <i>The importance of cell migration</i> .....	22
1.4. <i>RhoGTPases: family members and regulation</i> .....	25
1.4.1. RhoGTPases family members are regulators for cytoskeleton dynamics and cell migration .....	28
1.4.2. The atypical RhoGTPases family and tumourigenesis .....	32
1.5. <i>p21 activated kinase (PAK)</i> .....	37
1.5.1. PAKs overview .....	37
1.5.2. Domain architecture of PAKs .....	38
1.5.3. Regulation of PAK activity .....	42
1.5.4. PAK expression in cancer .....	44
1.5.5. Group II PAKs: Cancer cell metastasis .....	45
1.5.6. PAK6 .....	49
1.6. <i>FilaminA</i> .....	50
1.6.1. Biological function of Filamins .....	53
1.6.2. FilaminA in Cancer Invasion and Metastasis .....	56
1.7. <i>Aims of the project</i> .....	58
2. CHAPTER 2: MATERIALS AND METHODS .....	60
2.1. <i>Materials</i> .....	60
2.1.1. General Materials .....	60
2.1.2. Primary Antibodies .....	63
2.1.3. Secondary Antibodies .....	64
2.1.4. Plasmids .....	64
2.2. <i>Methods: Molecular Biology</i> .....	65
2.2.1. Transformation of <i>Escherichia coli</i> cells .....	65
2.2.2. Purification of plasmid DNA .....	65
2.2.3. Determination of DNA concentration .....	65
2.3. <i>Methods: cell biology</i> .....	66
2.3.1. Cell culture .....	66
2.3.2. Thawing and freezing cells .....	66
2.3.3. Lipofectamine transfection .....	67
2.3.4. Calcium Phosphate Transfection .....	68
2.3.5. HiPerfect siRNA transfection .....	68
2.3.6. Inhibitors .....	69
2.3.7. MTT Proliferation Assay .....	69
2.3.8. 3D Spheroid Invasion Assay .....	69
2.3.9. Preparation of coverslips for Immunofluorescence .....	70
2.3.9.1. Rat tail collagen type I coated coverslips .....	70
2.3.9.2. Fibronectin coated coverslips .....	70
2.3.10. Immunofluorescent labelling .....	70
2.3.11. Growth factor stimulation .....	71
2.3.11.1. Stimulation of cells on 6-wells plates .....	71
2.3.11.2. Stimulation of cells on coverslips .....	71
2.3.12. Image processing and Cell shape analysis .....	72
2.4. <i>Methods: Cell Biochemistry</i> .....	72
2.4.1. Preparation of cell lysates .....	72

2.4.2.	Gel electrophoresis and immunoblotting.....	72
2.4.3.	Stripping of Nitrocellulose Membranes .....	73
2.4.4.	Immunoprecipitation .....	73
2.4.5.	GFP-TRAP .....	73
2.4.6.	Densitometry .....	74
2.4.7.	Statistics.....	74
3.	CHAPTER 3: INVESTIGATION THE ROLE OF PAK6 IN BREAST CANCER .....	76
3.1.	<i>Introduction</i> .....	76
3.2.	<i>Results</i> .....	78
3.2.1.	PAK6 is endogenously expressed in MDA-MB-231 and MCF-7 cells .....	78
3.2.2.	Expression of growth factor receptors in panel of cancer cell lines.....	80
3.2.3.	Growth factor stimulation did not modify PAK6 autophosphorylation.....	83
3.2.4.	Cell morphology of breast cancer cell lines.....	88
3.2.5.	Growth factor mediated cell morphology changes .....	88
3.2.6.	PAK6 induces cell elongation in MCF-7 cells but not in MDA-MB-231 cells.....	92
3.2.7.	Overexpressing active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells.....	96
3.3.	<i>Discussion:</i> .....	103
3.4.	<i>Future Work</i> .....	107
4.	CHAPTER 4: CHARACTERISATION OF CELL MORPHOLOGY IN PAK6 MODULATED CELLS.....	109
4.1.	<i>Introduction</i> .....	109
4.2.	<i>Results:</i> .....	111
4.2.1.	PAK6 knockdown in MDA-MB-231 cells .....	111
4.2.2.	Depletion of PAK6 has no significant impact on MDA-MB-231 cell proliferation.....	111
4.2.3.	PAK6 depletion induces cell elongation .....	116
4.2.4.	LBH inhibitor reduces PAK6 expression .....	119
4.2.5.	LBH treatment phenocopies PAK6 knockdown cell morphology .....	120
4.2.6.	Active PAK6 can revert the LBH phenotype .....	124
4.2.7.	RhoA and ROCK but not Blebbistatin inhibitors prevent active PAK6 induced cell rounding .....	126
4.2.8.	Optimization the 3D spheroid invasion assay using MDA-MB-231 cells .....	131
4.2.9.	PAK6 is localised at the cell periphery in MDA-MB-231 cells.....	134
4.3.	<i>Discussion</i> .....	136
4.4.	<i>Future works</i> .....	141
5.	CHAPTER 5: IDENTIFICATION PAK6 BINDING PARTNERS .....	143
5.1.	<i>Introduction</i> .....	143
5.2.	<i>Results:</i> .....	145
5.2.1.	PAK6 interacts with RhoV, RhoU and RhoD.....	145
5.2.2.	MDA-MB-231 cell morphology is affected by RhoV and RhoD .....	151
5.2.3.	Identification of novel PAK6 binding partners by mass spectrometry .....	153
5.2.4.	FilaminA, MyosinIIA and 14-3-3 proteins interact with PAK6 .....	157
5.2.5.	Full-length PAK6 interacts with FilaminA .....	158
5.2.6.	FilaminA is expressed endogenously in breast cancer cell lines .....	160
5.2.7.	Co-expression of FilaminA and PAK6 induces morphological changes in MDA-MB-231 cells.....	164
5.2.8.	FilaminA phosphorylation levels are elevated in the presence of PAK6 in MDA-MB-231 cells.....	167
5.3.	<i>Discussion:</i> .....	169
5.4.	<i>Future work</i> .....	173
6.	CHAPTER 6: CONCLUDING REMARKS .....	175
7.	CHAPTER 7: APPENDIX .....	181
7.1.	<i>Generation of PAK6 isoform specific rabbit polyclonal antibody</i> .....	181
7.2.	<i>Validation of the in-house PAK6 antibody</i> .....	184
	<b>REFERENCES:</b> .....	<b>186</b>

## List of Figure

<b>Figure 1.1:</b>	A section of normal mammary gland duct	18
<b>Figure 1.2:</b>	Metastatic cascade of breast cancer	20
<b>Figure 1.3:</b>	Mechanisms of single cell or collective cell migration	24
<b>Figure 1.4:</b>	RhoGTPase family tree	26
<b>Figure 1.5:</b>	Regulation of RhoGTPase activity	27
<b>Figure 1.6:</b>	RhoGTPases regulate cell migration	31
<b>Figure 1.7:</b>	Interplay between atypical and classical Rho-family GTPases during cell migration.	33
<b>Figure 1.8:</b>	PAK family member and structures	40
<b>Figure 1.9:</b>	Group II PAKs mediated signalling pathways.	48
<b>Figure 1.10:</b>	The structure of FilaminA	52
<b>Figure 1.11:</b>	Diagram illustrating Filamin interactions	55
<b>Figure 3.1:</b>	PAK6 expression in breast and prostate cell lines	79
<b>Figure 3.2:</b>	c-Met expression in panel of cancer cell lines	81
<b>Figure 3.3:</b>	EGFR expression in panel of cancer cell lines	82
<b>Figure 3.4:</b>	HGF stimulation has no effect on PAK6 autophosphorylation levels in MDA-MB-231 cell line	84
<b>Figure 3.5:</b>	EGF stimulation has no effect on PAK6 autophosphorylation levels in MDA-MB-231 cell lines	85
<b>Figure 3.6:</b>	HGF stimulation has no effect on PAK6 autophosphorylation levels in MCF-7 cell line	86
<b>Figure 3.7:</b>	EGF stimulation has no effect on PAK6 autophosphorylation levels in MCF-7	87
<b>Figure 3.8:</b>	MCF-7 and MDA-MB-231 cell morphology	89
<b>Figure 3.9:</b>	Changes in cell morphology in MCF-7 cells downstream HGF but not EGF stimulation	90
<b>Figure 3.10:</b>	Changes in cell morphology in MDA-MB-231 cells downstream EGF but not HGF stimulation	91
<b>Figure 3.11:</b>	PAK6 overexpression in MDA-MB-231 cells	93

<b>Figure 3.12:</b>	Overexpression wild type PAK6 does not induce changes in cell morphology in MDA-MB-231 cells	94
<b>Figure 3.13:</b>	Overexpression wild type PAK6 induces changes in cell morphology in MCF-7 cells	95
<b>Figure 3.14:</b>	Overexpressing PAK6 mutants in MDA-MB-231 cell	98
<b>Figure 3.15:</b>	Overexpressing the active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells	99
<b>Figure 3.16:</b>	Overexpressing the active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells	100
<b>Figure 3.17:</b>	Overexpressing the active form of PAK6 (S531N) in MDA-MB-231 cells seeded on collagen and fibronectin	101
<b>Figure 3.18:</b>	Overexpressing the active form of PAK6 (S531N) in MDA-MB-231 cells seeded on collagen and fibronectin	102
<b>Figure 4.1:</b>	PAK6 knockdown in MDA-MB-231 cells	112
<b>Figure 4.2:</b>	PAK6 knockdown does not effect MDA-MB-231 cells after 48 and 72 hours	113
<b>Figure 4.3:</b>	Depletion of PAK6 has no significant impact on MDA-MB-231 cell proliferation	115
<b>Figure 4.4:</b>	PAK6 knockdown induce cell elongation in MDA-MB-231 cells	117
<b>Figure 4.5:</b>	PAK6 knockdown induce cell elongation in MDA-MB-231 cells	118
<b>Figure 4.6:</b>	LBH inhibitor reduces PAK6 expression	121
<b>Figure 4.7:</b>	The effect of LBH inhibitor on MDA-MB-231 cell proliferation	122
<b>Figure 4.8:</b>	LBH inhibitor phenocopies PAK6 knockdown cell morphology	123
<b>Figure 4.9:</b>	Overexpress active PAK6 revert the effect of LBH inhibitor	125
<b>Figure 4.10:</b>	PAK6 overexpression at different time points	127
<b>Figure 4.11:</b>	RhoA inhibitor prevents active PAK6 induce cell rounding	128
<b>Figure 4.12:</b>	ROCK inhibitor prevents active PAK6 induce cell rounding	129
<b>Figure 4.13:</b>	Blebbistatin does not prevent active PAK6 induce cell rounding	130
<b>Figure 4.14:</b>	Optimization of a 3D spheroid invasion assay using MDA-MB-231 cells	132
<b>Figure 4.15:</b>	3D spheroid invasion assay	133



<b>Figure 4.16:</b>	Localisation of exogenous PAK6 in MDA-MB-231 cells	135
<b>Figure 5.1:</b>	Co-immunoprecipitation of PAK6 with Cdc42, RhoV and RhoG	147
<b>Figure 5.2:</b>	RhoV and RhoU binds to PAK6	148
<b>Figure 5.3:</b>	Co-immunoprecipitation of PAK6 with RhoD	149
<b>Figure 5.4:</b>	RhoD binds to PAK6	150
<b>Figure 5.5:</b>	RhoD and RhoV overexpression induces morphological changes in MDA-MB-231 cells	152
<b>Figure 5.6:</b>	PAK6 immunoprecipitated from MDA-MB-231 cells	155
<b>Figure 5.7:</b>	Identification of novel PAK6 binding partners expressed in MDA-MB-231 cells by mass spectrometry	156
<b>Figure 5.8:</b>	PAK6 interacts with FilaminA in HEK293 cells	159
<b>Figure 5.9:</b>	Testing of FilaminA antibodies for detecting exogenous FilaminA	161
<b>Figure 5.10:</b>	FilaminA expression in panel of breast cell lines	162
<b>Figure 5.11:</b>	PAK6 interacts with FilaminA in MDA-MB-231 cells	163
<b>Figure 5.12:</b>	FilaminA overexpression does not induce morphological changes in MDA-MB-231 cells	165
<b>Figure 5.13:</b>	FilaminA co-expression with PAK6 induces morphological changes in MDA-MB-231 cells	166
<b>Figure 5.14:</b>	Active PAK6 expression significantly increases FilaminA phosphorylation levels	168
<b>Figure 6.1:</b>	Proposed model of PAK6 and FilaminA binding partner in regulation the change of cell morphology in breast cancer	179
<b>Figure 7.1:</b>	FilaminA expression in panel of breast cell lines	183
<b>Figure 7.2:</b>	PAK6 interacts with FilaminA in MDA-MB-231 cells	185

## List of Tables

<b>Table 1.1:</b> PAKs substrates	41
<b>Table 2.1:</b> General Materials	60
<b>Table 2.2:</b> Buffers and solutions	62
<b>Table 2.3:</b> Primers Antibodies	63
<b>Table 2.4:</b> Primary Antibodies	64
<b>Table 2.5:</b> Plasmid Constructs	64
<b>Table 2.6:</b> Lipofectamine transfection reaction mix	67
<b>Table 2.7:</b> Calcium phosphate transfection reaction mix	68
<b>Table 2.8:</b> The Sequences of siRNAs used in this study	69
<b>Table 3.1:</b> Panel of breast and prostate cell lines	78
<b>Table 5.1:</b> The results from mass spectrometry	157
<b>Table 7.1:</b> The sequences of PAK6	182

## List of Abbreviations

2D	2-Dimensional
3D	3-Dimensional
AID	Autoinhibitory domain
APS	Ammonium persulfate
AR	Androgen receptor
BLAST	Basic Local Alignment Search Tool
BM	Basement membrane
Cdc42	Cell division control protein-42
CRIB	Cdc42/Rac interactive binding region
DAPI	4', 6-diamidino-2-phenylindole
DCIS	Ductal carcinoma <i>in situ</i>
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DLC1	Dynein light chain 1
ECL	Enhance chemiluminescence
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK1	Extracellular receptor kinase 1
ERK2	Extracellular receptor kinase 2
F-actin	Filamentous actin
FBS	Foetal Bovine Serum
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
G-actin	Globular actin

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAPs	GTPase activating proteins
GBD	GTPase binding domain
GEF	Guanine nucleotide exchange factor
GDI	Dissociation inhibitors
GDP	Guanosine diphosphate
GID	Gab1 interacting domain
GTPase	activating protein
HCC	Hepatocellular carcinoma
HEK	Human Embryonic Kidney
HER2	HER Human epidermal receptor-2
HCL	Hydrochloric acid
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
IBC	Invasive breast cancer
IGFR	Insulin-like growth factor 1 receptor
IP	Immunoprecipitation
IQGAP1	IQ motif containing GAP
JNK	C-Jun N-terminal Kinase
LB-agar	Luria-Bertani agar
LB-broth	Luria-Bertani broth
LBD	Ligand-binding domain
LCIS	Lobular carcinoma <i>in situ</i>
LiCl	Lithium chloride
LIMK	LIM kinase
MAPK	Mitogen activated protein kinase
MgCl <sub>2</sub>	Magnesium chloride
MLC-2	Myosin light chain-2
MKK6	Mitogen activated protein kinase kinase 6
MMPs	Matrix metalloprotease
MTT	Methyl thiazolyldiphenyl tetrazolium bromide

Na <sub>3</sub> VO <sub>4</sub>	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOH	Sodium hydroxide
OS	Overall survival
PAKs	p21-activated kinases
PBS	Dulbecco's phosphate buffered saline
PFA	Paraformaldehyde
PIX	PAK-interacting exchange factor
PR	Progesterone receptor
RFP	Red fluorescent protein
ROCK	Rho associated protein kinase
RSK	Ribosomal S6 Kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering RNA
TEMED	Tetramethylethylenediamine
TKIs	Tyrosine kinase inhibitors
TRITC	Tetramethylrhodamine isothiocyanate - Phalloidin
UT	Untransfected
WCL	Whole cell lysates
WT	Wild type

# **Chapter 1:**

## **Introduction**

## **1. Chapter 1: Introduction**

### **1.1. Breast cancer**

Breast cancer is a complex and heterogeneous disease and despite the recent advances in the understanding of the mechanisms of breast cancer progression and novel therapeutics, it remains the most common cause of cancer morbidity in women worldwide (Siegel et al., 2012, Jemal et al., 2011). Approximately, 10-15% of diagnosed breast cancer patients develop distant metastasis within three years (Weigelt et al., 2005). Multiple factors can influence the development of breast cancer and these include obesity, excessive alcohol consumption, limited physical activity and/or the use of oral contraceptives and hormonal therapy for menopause treatment (Marchbanks et al., 2012, Shin et al., 2010, Hortobagyi et al., 2005, Parkin and Fernández, 2006, Youlden et al., 2012). In addition, genetics can also play a prominent role as a risk factor for breast cancer. In 65%-80% of hereditary breast cancers, there are mutations in the tumour suppressor genes BRCA-1 and BRCA-2 that increase the risk of developing the disease (Ripperger et al., 2008).

Breast cancer subtypes can be defined by the expression of different combinations of markers. The expression of progesterone receptor (PR) and estrogen receptor (ER) is commonly referred to as hormone receptor positive disease. Some patients exhibit ERBB2-amplification (also known as human epidermal receptor-2 (HER2)). Alternatively, some patients are classified as triple negative as they lack the expression of PRs, ERs and HER2 (Lorusso and Ruegg, 2012). An increased understanding of the biology of breast cancer has resulted in the identification of a number of potential molecular targets and the development of novel therapeutics. These include tyrosine kinase inhibitors (TKIs) directed towards different targets such as HER1, HER2, c-MET, FGF receptor (FGFR), IGF receptor (IGFR), intracellular signalling pathways (PI3K, AKT, ERK) as well as angiogenesis inhibitors (Higgins and Baselga, 2011). Some of these drugs are already being used in the clinic to treat patients with breast cancer; for example, the anti-HER2 agents trastuzumab and lapatinib (Higgins and Baselga, 2011).

Despite trastuzumab being an effective treatment in early stage HER2 breast cancer, the majority of patients with advanced HER2 breast cancers develop trastuzumab resistance.

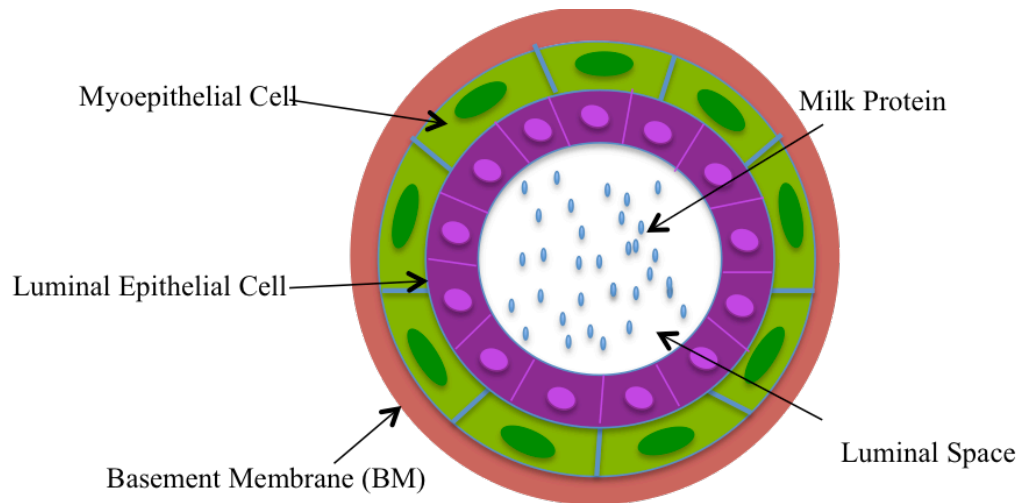
Trastuzumab resistance promotes epithelial to mesenchymal transition (EMT) transforming HER2+ disease into a triple negative breast cancer that requires additional treatment options (Higgins and Baselga, 2011). Although advances in the treatment of breast cancer have significantly enhanced the survival of patients, further treatment options for metastatic breast cancer are required, particularly, the identification of new therapeutic strategies that target metastatic recurrence and chemoresistance.

### **1.1.1. Breast Cancer Progression**

Normal human breast glands contain a branching ductal-lobular system, which consists of two epithelial cell types. The outer layer of myoepithelial cells and the inner layer of luminal epithelial cells are separated from the interstitial stroma by an intact basement membrane (BM) (Figure 1.1) (Adriance et al., 2005). Myoepithelial cells form a natural border to separate proliferating epithelial cells from the BM and underlying stroma (Deugnier et al., 2002). The disruption of both the BM and the myoepithelial cell layer promote breast tumour invasion progression and metastasis (Man and Sang, 2004, Man, 2007, Gudjonsson et al., 2002).

Breast tumours are mainly found to be epithelial in origin and arise from the terminal ductal lobular unit. The disease progresses from an atypical epithelial hyperplasia into either ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS). In-situ carcinoma is defined as the presence of proliferating tumour cells that have undergone malignant transformation but remain at their site of origin, confined by a basement membrane (Polyak, 2007, Sakorafas and Tsiotou, 2000, Sakorafas et al., 2008). Invasive breast cancer (IBC) is described as a loss of myoepithelial cell layer and breakdown of the basement membrane. At this stage the tumour cells invade surrounding stroma, intravasate local vasculature and migrate to distant sites; this process is termed as metastasis (Richie and Swanson, 2003).





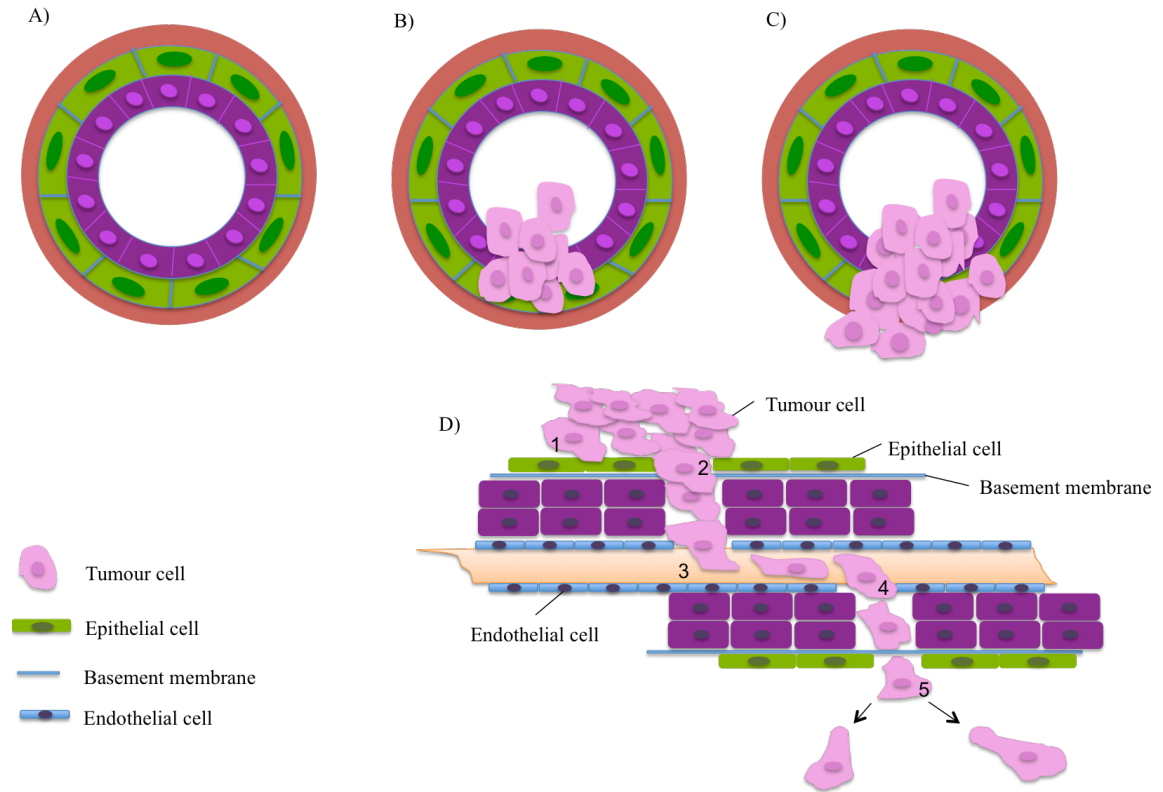
**Figure 1.1: A cross-section of normal mammary gland duct.** Normal breast ducts are composed of the basement membrane and a layer of luminal epithelial and myoepithelial cells.

The development of primary tumour and subsequent metastasis is now thought to involve the acquisition of multiple genetic alterations including both gene activation and inactivation over time (Weigelt et al., 2005). Moreover, more recently the tumour microenvironment has been implicated in driving cancer progression (Yoder et al., 2007). Taken together, these data reveal the extreme complexity involved in breast cancer progression and the need to gain a better understanding of the mechanisms that contribute to the development of various stages of the disease, including invasion, migration and metastasis.

## **1.2. Carcinoma Metastasis**

Metastatic disease is the most prevalent cause of cancer related mortality due to its systemic nature and the resistance of tumour cells to existing treatments. Tumour progression towards metastasis includes a series of changes in cell behaviour, driven by oncogenic transformation. Although there are many cellular processes that require cell migration, this study has focused on breast cancer cell migration. Breast cancer has the potential to spread to almost any region of the body and the most common secondary site is bone, followed by lung and liver (Weigelt et al., 2005). However, since the treatment opportunities for advanced metastatic breast cancer are limited, it is the late stage of the disease that accounts for the highest number of breast cancer deaths.

Primary tumours initiate as a consequence of multiple mutations and epigenetic changes altering key genes that affect cell proliferation and survival. In order for cells to metastasise, there are a number of key cellular step changes required including the detachment from the primary tumour, local invasion, intravasation into the blood or lymphatic system, dissemination and extravasation, and subsequent outgrowth at distal, secondary sites (Figure 1.2) (Valastyan and Weinberg, 2011, Yilmaz and Christofori, 2010).



**Figure 1.2: Metastatic cascade of breast cancer.** A) A section of normal mammary gland duct. B) *In situ* carcinomas where the abnormal cells are epigenetically and phenotypically transformed. C) Invasive carcinoma here breakdown of the basement membrane causes tumor cells to invade to surrounding tissues and migrate to distant organs; leading to micro metastases. D) Schematic representative magnification of epithelial carcinoma as a multistage process involving (1) aberrant growth and proliferation to form a primary tumour. (2) Tumour cells eventually acquire a migratory phenotype and invade through the basement membrane. (3) Tumour cells entering blood or lymphatic vessels by intravasation. (4) Tumour cells extravasate (5) Tumour cells invade, proliferate and subsequently form secondary tumours at distal sites around the body.

The ability of the disseminated cells to survive and proliferate at new sites is dependent on key interactions between the tumour cells and their microenvironment (Chambers et al., 2002). Improving knowledge of the basic biology at each step contributing to the metastatic process is crucial for improved therapeutic strategies (Figure 1.2).

As illustrated in Figure 1.2, the cancer cells must first break away from the primary tumour, often undergoing epithelial mesenchymal transition (EMT). One of the key features of metastasis is that tumour cells transform from non-invasive cells to migrating, metastatic cancer cells. This transition process is initiated when the cells acquire the ability to dissociate from intercellular adhesions and become motile (Friedl and Wolf, 2003), which is usually driven by complex regulatory signalling cascades that transiently and/or permanently alter the expression of multiple proteins that act to reorganise the cytoskeletal network (Yamazaki et al., 2005). Down regulation of E-cadherin via aberrant transcriptional regulators, such as Snail and Twist is a hallmark of tumour progression leading to EMT by disturbing cell-cell adhesion (Hanahan, 2000, Vandewalle et al., 2005, Brabek et al., 2010, Hanahan and Weinberg, 2011). Such changes may then allow the cells to implement a more motile phenotype (Yilmaz and Christofori, 2010).

In order to spread, the cancer cells must invade through the basement membrane (Hotary et al., 2006, Ihara et al., 2011), which involves the production of extracellular proteases (Deryugina and Quigley, 2006). Matrix metalloprotease (MMPs) are the most prominent protease that permits cancer cells to metastasise and whilst basement membrane and extra cellular matrix ECM degradation is their primary function, MMPs also regulate the pathways associated with cell growth and angiogenesis (Kessenbrock et al., 2010, Wolf et al., 2007). Previous reports have suggested that there is an increase in the expression of MMP9 in breast cancer tissues compared with normal breast tissues (Scorilas et al., 2001, Wu and Frost, 2006, Wu et al., 2008, Bottino et al., 2014). Moreover, a meta-analysis study demonstrated that MMP9 overexpression could act as a biomarker suggesting unfavorable results on both overall survival (OS) and relapse-free survival (Song et al., 2013).

Once the basement membrane has been penetrated, cancer cells can invade locally and disseminate from the primary tumour and enter the stroma (Valastyan and Weinberg, 2011).

Thus, this allows the malignant cells to migrate to a different site.

### **1.3. The importance of cell migration**

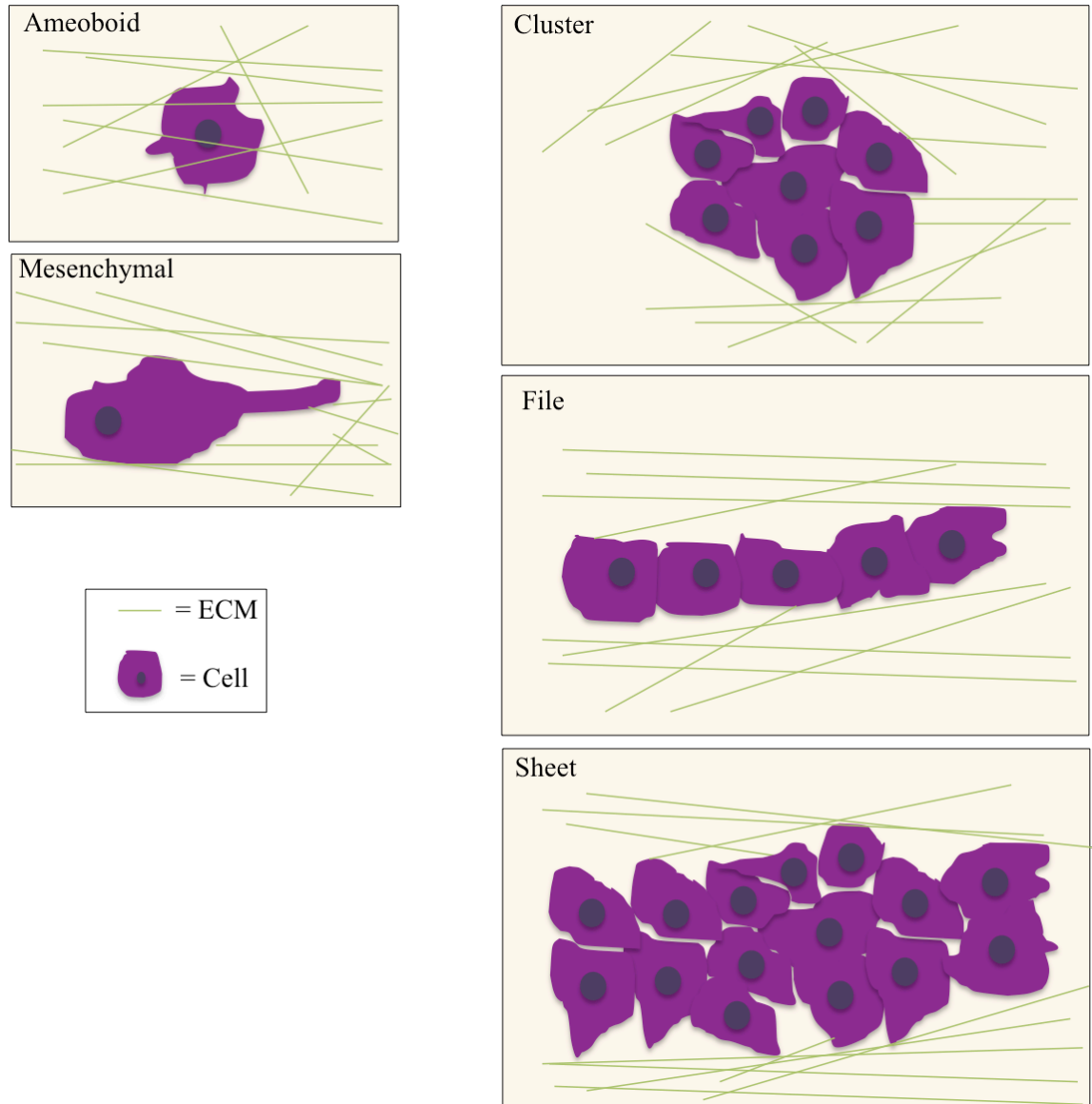
Cell migration is a critical process in all-multicellular organisms and plays a significant role in carcinoma metastasis (Chaffer and Weinberg, 2011). Thus, fundamental mechanisms underlying cell migration are likely to provide suitable targets for effective therapeutic approaches for treating disease. Over the past few years, improvement has been made in understanding cell migration, including the establishment of polar structures and the mechanisms involved in regulating the dynamic process of actin and microtubule polymerisation (Ridley et al., 2003, Zigmond, 1996).

Cancer cells can migrate through the ECM via two methods, either as individual cells (termed as single cells migration) or as a collective group (termed as collective migration). Single cell migration has two alternative mechanisms described as mesenchymal or amoeboid migration (Figure 1.3). The cells moving via mesenchymal type migration have a characteristic elongated morphology and extended long protrusions at their front. Molecules such as MMPs are recruited to aid in developing a pathway through the dense ECM meshwork (Brabek et al., 2010). In contrast, during amoeboid migration, cells have a rounded shape. This type of cell migration requires the activation of the Rho/ROCK pathway and subsequent phosphorylation of myosin light chain-2 (MLC-2) (Brabek et al., 2010, Sahai and Marshall, 2003, Webb and Horwitz, 2003).

Three hallmarks characterize collective cell migration. First, the cells remain functionally and physically connected, which means that cells maintain their cell-cell junctions and migrate in cell clusters, files and sheets as shown in Figure 1.3, either separated or still in connection with their originating tissue (Sahai, 2005). Second, multicellular polarity and the organization of the actin cytoskeleton produce protrusion force and traction for migration. Third, in most modes of collective migration, moving cell groups structurally modify the tissue along the migration path, either by causing secondary ECM modification, involving the deposition of a basement membrane or by clearing the track. Cells invading collectively can adopt different morphologies, which depend on the number of cells, cell type and the structure of the tissue that is being invaded (Friedl and Alexander, 2011, Geiger and Peeper,

2009). In cancer, collective cell migration and invasion is found in different cancer types, including high and intermediate differentiated types of lobular breast cancer, melanoma, large cell lung cancer, prostate cancer, and most prominently in squamous cell carcinoma. In the most type of collective invasion, there are leader cells producing MMPs, which show similar characteristics to mesenchymal migration type cells and form the leading edge of multicellular strands, generating a forward traction and pericellular proteolysis of the invaded tissue. High-resolution multimodal microscopy has revealed that the guiding cells use  $\beta$ 1-integrin which mediated focal adhesions and at their leading edges expression of MT1-MMP to cleave collagen fibers and orient them in a way that generates tube like microtracks into which the collective mass migration of follower cells can occur. There is also described a less frequent type of collective invasion, where cells with strong epithelial polarity in soft tissues aggregate in a tip of multiple cells that protrude along the tissue space but change position, therefore lacking clear defined leader cells (Friedl and Alexander, 2011, Friedl et al., 1995, Wolf et al., 2007, Friedl and Wolf, 2008).

A variety of intracellular signalling molecules are associated with cell migration, including mitogen activated protein kinase (MAPK) cascades, phospholipases, lipid kinases, serine/threonine or tyrosine kinases and scaffold proteins. Invasive cell migration requires the coordinated regulation of both actin cytoskeletal rearrangements and regulation of adhesive interactions with the microenvironment processes that are known to be regulated by Rho family GTPases (Vega and Ridley, 2008).



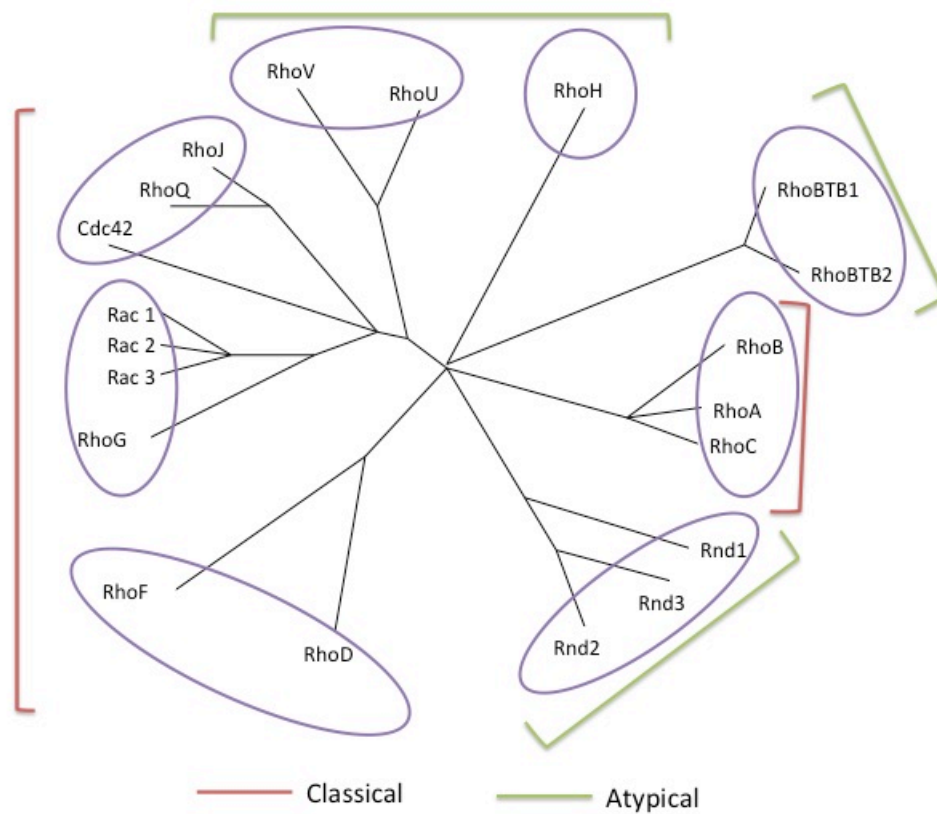
**Figure 1.3: Mechanisms of single cell or collective cell migration.** Cells can migrate either individually or collectively via different mechanisms. Single cell migration can be separated into amoeboid cell movement involving rounded and highly contractile cells or mesenchymal cells, which have an elongated shape and use protease degradation. In collective cell migration a group of cells migrate in either a small cluster, file or a sheet. Figure is adapted from (Friedl and Wolf, 2003).

#### **1.4.RhoGTPases: family members and regulation**

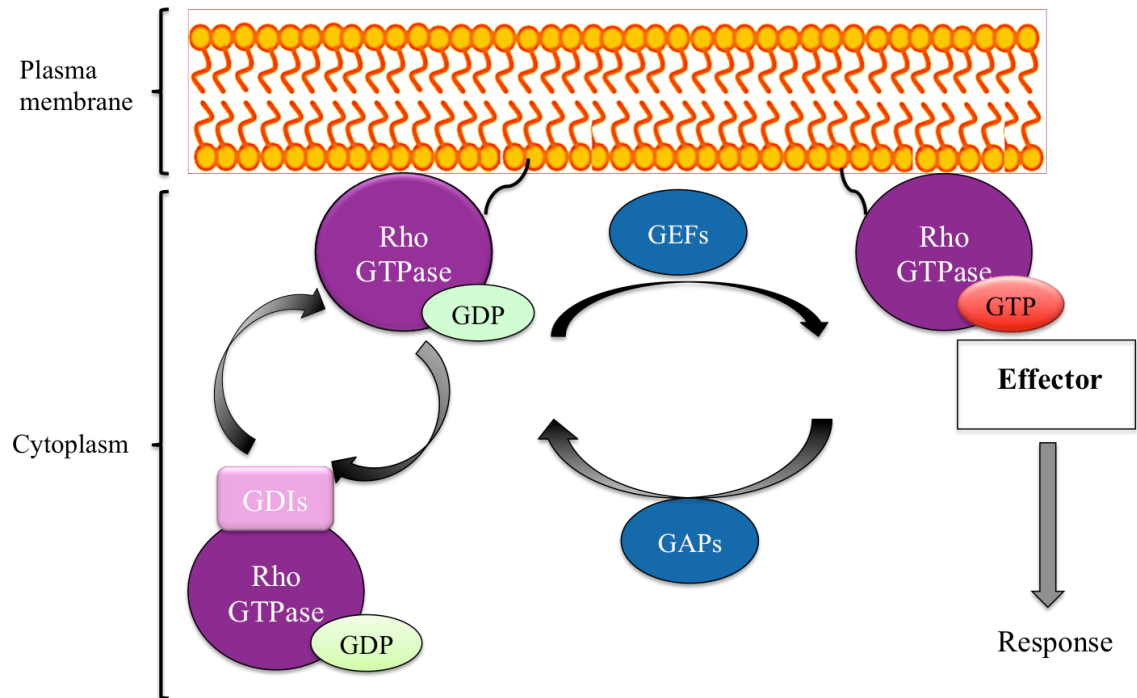
RhoGTPases form a distinct family within the Ras-like protein superfamily, which also includes the Ras, Rab, Ran and Arf families (Vega and Ridley, 2008). Rho members differ from other Ras-like GTPases due to the presence of a Rho-specific insert domain. RhoGTPases are ubiquitously expressed. In mammals, 20 members have been identified and divided into eight different subfamilies: the RhoA, B and C subfamily, the Cdc42, the RhoJ and RhoQ subfamily, the Rac1, 2, 3 and RhoG subfamily, the RhoV and RhoU subfamily, the RhoD and RhoF subfamily, the RhoH subfamily, the Rnd1, 2 and 3 subfamily, and finally the RhoBTB 1 and 2 subfamily (Figure 1.4). Out of these subfamilies, Rnd, RhoU, RhoH, RhoBTB and RhoV, have characteristics that make them atypical compared to the classical RhoGTPase Rho, Rac, Cdc42 and RhoD/RhoF subfamilies (Aspenström et al., 2007, Ridley, 2013).

The RhoGTPase subfamilies can be classified into two distinct types of RhoGTPases, the classical and the atypical RhoGTPase. Most classical Rho family members, including the Rho, Rac, Cdc42, RhoF and RhoD subfamilies, act as molecular switches to control signal transduction pathways through cycling between a GDP-bound inactive form and a GTP-bound active form (Vega and Ridley, 2008). The RhoGTPases cycle is regulated by three groups of proteins; the Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP to activate a GTPase, the GTPase activating proteins (GAPs) negatively regulate the switch by converting the proteins to the GDP-bound inactive conformation, and guanine nucleotide dissociation inhibitors (GDIs) bind to some but not all RhoGTPases, and prevent their interaction with the membrane by hiding the prenyl group as well as inhibiting these proteins from binding to downstream targets (Figure 1.5) (Ridley, 2006).





**Figure 1.4: RhoGTPase family tree.** RhoGTPases are a family of 20 proteins, divided into 8 subfamilies. These families can be classified as classical or atypical, depending on their type of regulation. Figure is adapted from (Vega and Ridley, 2008).



**Figure 1.5: Regulation of RhoGTPase activity.** RhoGTPases cycle between an inactivate GDP bound and an active GTP bound form. RhoGTPases are activated via GEFs and inactivated via GAPs. The binding of GDIs, which sequester the GTPase in the cytoplasm, can also inhibit GTPase activation.

Consequently, GEFs, GAPs and GDIs play key roles in regulating classical Rho GTPases. However, the atypical members of the GTPase family infrequently shadow the simple scheme illustrated above and there is little evidence for the existence of GEFs or GAPs for atypical RhoGTPases. These proteins are permanently bound to GTP (Aspenström et al., 2007, Chardin, 2006, Wennerberg, 2004), and their functions are likely to be regulated by protein:protein interactions, including types of domains that are absent in classical Rho proteins.

Once activated, RhoGTPases interact with cellular target protein effectors to stimulate a variety of intracellular response (Bishop and Hall, 2000). More than 40 effectors, 80 GEFs and 80 GAPs have been described for the mammalian Rho family (Raftopoulou and Hall, 2004, Tcherkezian and Lamarche-Vane, 2007, Ridley, 2016). RhoGTPases are also involved in regulating the dynamics of the actin cytoskeleton and provide a key signalling link through which spreading, adhesion and migration are organised and regulated (Lawson and Burridge, 2014).

#### **1.4.1. RhoGTPases family members are regulators for cytoskeleton dynamics and cell migration**

The Rho family GTPases Cdc42, RhoA and Rac1 are well characterised in their ability to control actin polymerisation dynamics, and this is a fundamental requirement for cell migration. The actin cytoskeleton is responsible for regulating changes in cell shape as well as the generation of forces required for cells to migrate (Hall, 1998). In initial Rho GTPase studies, Swiss 3T3 cells were microinjected with activated RhoA to induce changes in cell morphology (Paterson et al., 1990). Subsequently, it was demonstrated that in fibroblasts, active mutants of RhoA and Rac1 prompt the generation of stress fibres or lamellipodia respectively (Ridley, 1992a, Ridley, 1992b). Lamellipodia that are generated by Rac1 can be recognised by their flat and broad morphology. Further studies revealed a role for Cdc42 in the induction of filopodia (Kozma et al., 1995, Nobes and Hall, 1995), which are characterised as finger like projections. Thus, RhoA, Rac1 and Cdc42 are central in remodelling actin during cell movement (Figure 1.6). As cell migration is a pre-requisite for cancer cell invasion, these RhoGTPases have been linked with cancer cell metastasis (Ellenbroek and Collard, 2007).

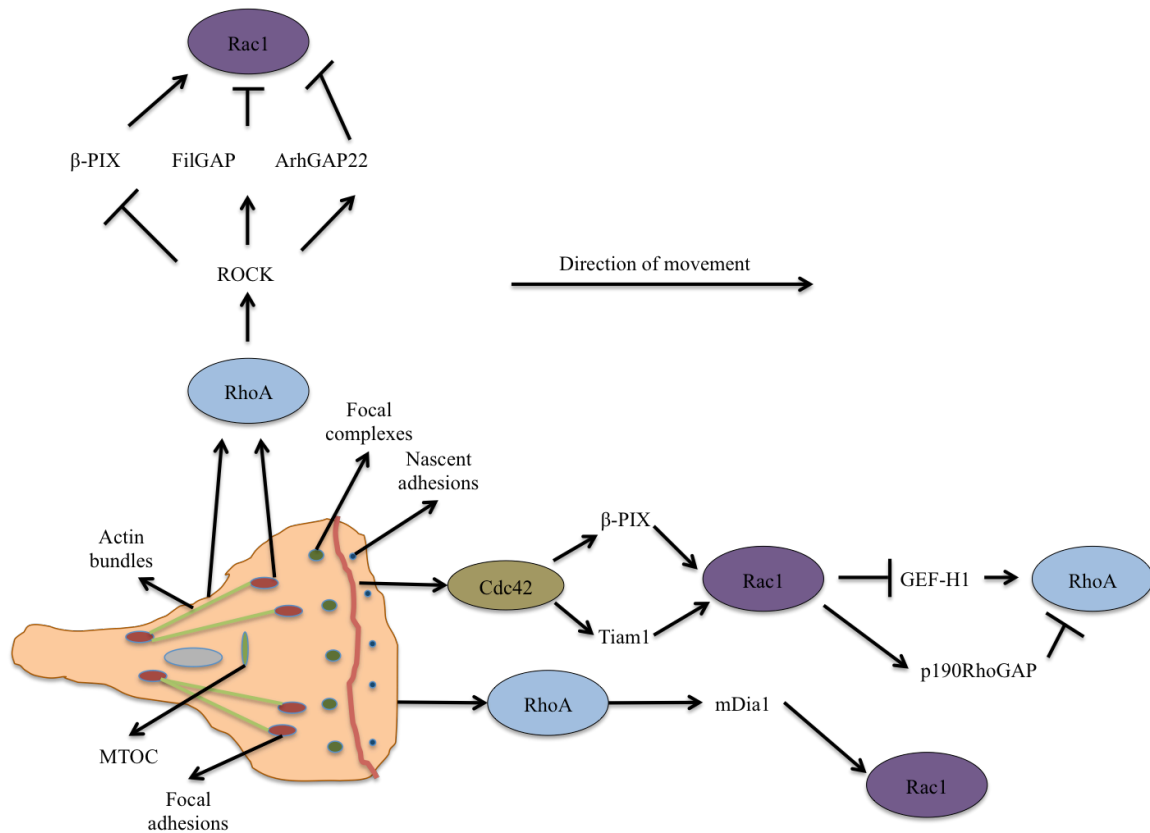
Although Rac1, Cdc42 and RhoA localise to lamellipodia protrusions (Machacek et al., 2009), the subsequent response is dependent on their activation status, which may include cross talk between the proteins. Cdc42 can directly control Rac1 activation through PIX and Tiam1, which are both Rac GEFs (Li et al., 2003, Cau and Hall, 2005, Pegtel et al., 2007). Rac1 can inhibit the activation of RhoA via PAK induced inhibition of RhoAGEFs, including GEF-H1. Indeed, Rac1 and RhoA are mutually inhibitory proteins, with each able to inhibit the activity of the other (Sanz-Moreno et al., 2008, Machacek et al., 2009). The activation of ROCK through RhoA can activate LIM kinase (LIMK). While Rac and Cdc42, can activate LIMK via PAK activation, activated LIMK phosphorylates and inhibits the activation of cofilin, a protein that can promote actin depolymerization (Edwards et al., 1999, Stanyon and Bernard, 1999). This suggests that Rac could inhibit cofilin-induced depolymerization. However, it is also shown that cofilin is required for and promotes lamellipodium extension and cell migration (Aizawa, 1996, Chen et al., 2001), either by severing actin filaments and thus providing more barbed ends for actin polymerization and/or by promoting release of actin monomers that can then be reincorporated into growing actin filaments at the plasma membrane (Ridley, 2001, Zebda et al., 2000).

The activation of Rac1 or RhoA or ROCK signalling pathways has been correlated with *in vitro* cell migration, as well as *in vivo* invasion and progression (Sahai et al., 2001, Itoh et al., 1999, Kaneko et al., 2002). Therefore, pharmacological inhibitors that block RhoA, ROCK or MLCK are being developed to interfere with cancer cell invasion (Somlyo et al., 2003, Itoh et al., 1999, Kaneko et al., 2002, Sahai et al., 2001). Given their role in driving cell migration, it is not surprising that Cdc42, Rac1 and RhoA all contribute to the mechanisms by which tumour cells migrate. Indeed, Cdc42, Rac1 and RhoA control the amoeboid or mesenchymal phenotype of single cell migration via the reorganisation of the cytoskeleton. Cdc42 has a role in both mesenchymal migration to polarise the cell, and amoeboid migration through PAK2 to increase MLC phosphorylation and actomyosin contractility (Gadea et al., 2008). Furthermore, Cdc42 is a key inducer of the invasive protrusion, invadopodia, which are thought to be used by tumour cells to invade through the ECM (Murphy and Courtneidge, 2011). In support of this notion, studies have demonstrated that the depletion of Cdc42 reduces the metastasis of breast cancer *in vivo* (Reymond et al., 2013). The activation of Rac1 promotes mesenchymal migration, which can be induced by

adaptor proteins such as NEDD9 or activators such as DOCK3 (Sanz-Moreno et al., 2008). Rac1 also inhibits the formation of an amoeboid cell shape by reducing actomyosin contractility (Sanz-Moreno et al., 2008). Moreover, cell invasion was also reduced by the depletion Rac1.

In contrast, the amoeboid mechanism of migration is highly dependent on RhoA (Sahai and Marshall, 2003, Orgaz et al., 2014). The amoeboid migration occurs via the activation of ROCK that stimulate myosin light chain 2 to enhance myosin which induces actin contraction (O'Connor and Chen, 2013). The signalling pathways eliciting amoeboid migration may also result in the inhibition of mesenchymal migration. Amoeboid migration occurs by the activation of ARHGAP22, which is stimulated by ROCK, and in turn, inhibits the activation of Rac1 (Sanz-Moreno et al., 2008). Moreover, the inhibition of blebbistatin and ROCK by the inhibitor (H1152) results in mesenchymal migration of elongated cells (Sanz-Moreno et al., 2008).

Antagonism between Rac1 and RhoA contributes to their polarising actions on cells (Meili and Firtel, 2003, Xu et al., 2003, Burridge and Wennerberg, 2004, Caron, 2003). Rac1 inhibits RhoA through its activation of p190RhoGAP (Nimnual et al., 2003). In addition, the inhibition of Rac1 activity can be dependent on raised RhoA activity, which is associated with an increase in actomyosin contractility. RhoA stimulation of ROCK mediated phosphorylation and activation of the FilaminA-associated protein FilGAP may contribute to the suppression of Rac1 activity (Figure 1.6) (Ohta et al., 2006, Shifrin et al., 2009). FilaminA links the cytoplasmic tails of  $\beta$  integrin subunits to actin, and external shear or myosin II-dependent force production can result in the dissociation of FilGAP from FilaminA (Ehrlicher et al., 2011). FilGAP has been shown to promote mesenchymal-to-amoeboid transition downstream of ROCK (Saito et al., 2012). Moreover, it is reported that RacGAP1 can inhibit Rac at the latter stages of adhesion (Jacquemet et al., 2013). This Rac-specific GAP is recruited to sites of  $\beta$ 1-integrin activation in the lamellipodia of spreading cells by a complex involving FilaminA and IQGAP1, which does not have intrinsic GAP activity (Jacquemet et al., 2013).



**Figure 1.6: Rho GTPase regulation of cell migration.** During cell migration Rho GTPases regulate activities in different areas of the cell. RhoA, Cdc42 and Rac1 are key examples of Rho GTPase family members participating in these processes. Both Cdc42 and Rac1 are activated at the leading edge of the cell, where Cdc42 establishes the polarity and directionality of cell movement, whilst activated Rac induces lamellipodium extension. In this region of the cell, both Cdc42 and Rac1 can suppress RhoA activity. RhoA activation initiates the formation of actin bundles or stress fibres, containing myosin II, which generate contractile forces via ROCK and myosin II. These forces lead to cell body contraction that coupled with the tail retraction allow the cell to move forward. Moreover, RhoA can suppress Rac1 activity, preventing the generation of actin polymerisation at the rear of the cell.

### **1.4.2. The atypical RhoGTPases family and tumourigenesis**

The atypical RhoGTPases do not generally appear to be regulated in the same way as classical RhoGTPases which are regulated by GTP–GDP cycling. Instead, the atypical RhoGTPases are constitutively GTP-bound, because they either have substitutions in their GTPase domain that prevent GTPase activity or possess high intrinsic nucleotide exchange activity (Hodge and Ridley, 2016). RhoU and RhoV have a high intrinsic nucleotide exchange rate *in vitro*. Whilst the Rnd (Rnd1, Rnd2 and Rnd3/RhoE), RhoH and RhoBTB proteins (RhoBTB1 and RhoBTB2) have amino acid substitutions that prevent them from hydrolysing GTP. These amino acid substitutions are at the equivalent amino acids that are mutated in Ras oncogenes, and known to prevent/decrease GTP hydrolysis. Furthermore, these atypical family members have unique N-terminal and C-terminal extensions to the standard G-domain (Borda-d’Agua et al., 2014). The subfamily including RhoF, RhoD, RhoQ, RhoJ are also considered to be atypical as they exhibit a high intrinsic exchange activity and therefore bound GTP under equilibrium and quiescent conditions (Jaiswal et al., 2013). During cell migration, there are interactions between atypical and classical Rho-family GTPases. RhoD, Rnd1, Rnd3, and RhoJ antagonize the Rho-mediated actin remodelling during cell migration. RhoG activates Rac by recruiting the ELMO/DOCK complex. RhoU promotes cell migration through activation of Rac, whereas RhoH antaintergonizes Rac-mediated actin reorganization and cell migration (Figure 1.7). RhoV also antagonizes cell migration by promoting PAK degradation. In endothelial cells, Rnd2 and Rnd3 promote RhoB-induced stress fiber formation. RhoD regulates the reorganization of actin through the activation of ZIPK and Arp2/3. RhoF activates mDia and is involved in the targeting of active ROCK to the cell cortex (Sadok and Marshall, 2014). In this thesis will be focused in RhoU and RhoV in more details.

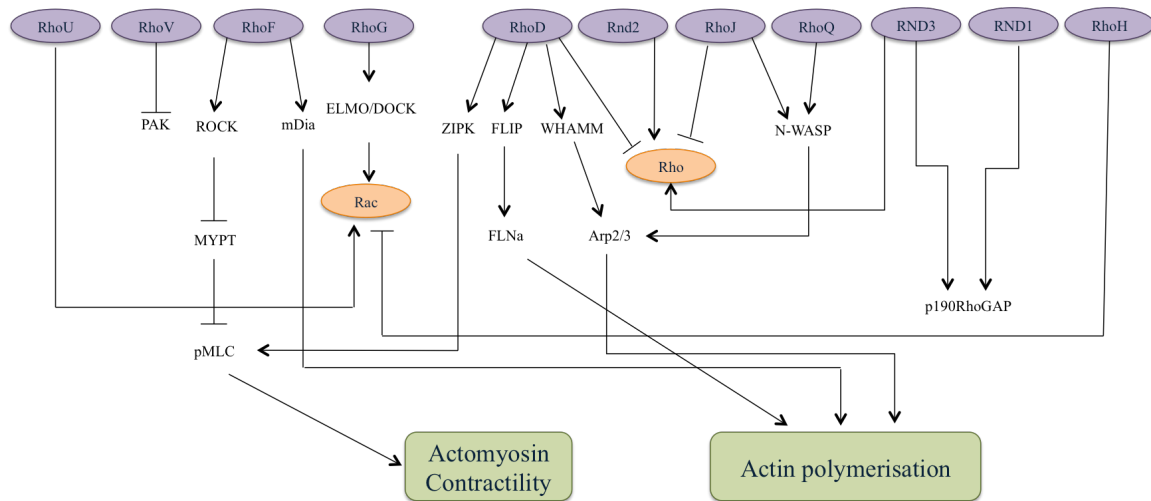


Figure 1.7: Interplay between atypical and classical Rho-family GTPases during cell migration. RhoU stimulates cell migration through activation of Rac, whereas RhoH antaintergonizes Rac-mediated actin reorganization and cell migration. RhoV also antagonizes cell migration by promoting PAK degradation. RhoF activates mDia and is involved in the targeting of active ROCK to the cell cortex. RhoD, Rnd1, Rnd3, and RhoJ antagonize the Rho-mediated actin remodelling during cell migration. Rnd2 and Rnd3 promote RhoB-induced stress fiber formation. RhoD regulates the reorganization of actin through the activation of Arp2/3 and ZiPK. RhoG activates Rac by recruiting the ELMO/DOCK complex. Adopted from (Sadok and Marshall, 2014).



RhoU (also known as Wrch1) was identified in 2001 as a Wnt-inducible gene (Tao et al., 2001), whereas RhoV (also known as Chp) was first discovered in 1998 as a p21-activated kinase-2 (PAK2) interacting protein (Aronheim et al., 1998). Although RhoU and RhoV share sequence similarities with Cdc42, unlike other Rho family members, both have a N-terminal proline-rich domain that is not present in other Rho family members. This domain can bind to SH3 domain-containing proteins such as Nck2 and Grb2, which increase RhoU activity (Aspenström et al., 2007, Shutes et al., 2004, Saras et al., 2004). Moreover, RhoU and RhoV are modified by palmitoylation but unlike other Rho family members they do not undergo isoprenylation (Berzat et al., 2005, Chenette et al., 2005b). Palmitoylation is a reversible process, which allows proteins to associate transiently with membranes, regulating their localisation and trafficking (Baekkeskov and Kanaani, 2009). RhoU and RhoV localisation are affected by inhibitors of protein palmitoylation but not by inhibitors of protein prenylation (Berzat et al., 2005, Chenette et al., 2005b).

RhoU and RhoV have been associated with roles in adhesion, migration, cell proliferation and transformation. Overexpression of RhoU or RhoV has been revealed to stimulate lamellipodial or filopodial extensions and/or integrin based focal adhesions (Aronheim et al., 1998, Aspenström et al., 2007, Chuang et al., 2007). RhoU localises to focal adhesions in HeLa cells and depletion of RhoU increased focal adhesion formation and inhibited cell migration (Chuang et al., 2007). PAK4 and RhoU cooperate to drive adhesion turnover and promote cell migration in MDA-MB-231 cells. Loss of RhoU expression reduces cell adhesion turnover and migration, whereas overexpression of RhoU rescues the PAK4 depletion phenotype (Dart et al., 2015). RhoU has also been localised to podosomes and is thought to influence integrin signalling in osteoclasts (Brazier et al., 2009). On the other hand, RhoU depletion reduced adhesion and migration in T-acute lymphoblastic leukaemia cells (Bhavsar et al., 2013). Similarly, neural crest cells were shown to adhere poorly when depleted of RhoU and had a rounded morphology (Fort et al., 2011). Therefore, RhoU plays an essential role in adhesion of cells to extracellular matrix, which impacts on cell migration but its specific function may be cell type specific.

RhoU is up regulated by the Wnt signalling pathway. Consequently, RhoU could be involved in Wnt-driven oncogenic transformation (Tao et al., 2001). It is reported that RhoU can be up regulated or down regulated in some primary tumours (Aspenström et al., 2007, Shutes et al., 2004) but it is not known whether this correlates with the levels of Wnt signalling. Overexpression of RhoU stimulates actin cytoskeletal reorganisation including the formation of filopodia and dissolution of stress fibres (Saras et al., 2004). Moreover, RhoU stimulates the cell cycle progression and the transformation of fibroblasts when overexpressed (Tao et al., 2001, Shutes et al., 2004). Whether this reflects RhoU physiological function or is just a consequence of sequence similarity to Cdc42 is not known (Vega and Ridley, 2008).

Several binding partners for RhoU have been identified including Grb2, NCK, FAK and PAR6. In pancreatic cancer cells upon epidermal growth factor (EGF) stimulation, RhoU colocalises with the EGF receptor on endosomes in a Grb2-dependent manner (Zhang et al., 2011). RhoU binds Pyk2 the non-receptor tyrosine kinase. This interaction requires RhoU to be in a GTP-bound form and also involves the N-terminal proline-rich extension. Moreover, The interaction depends on the presence and activity of Src and has a role in filopodium formation in fibroblasts.(Ruusala and Aspenstrom, 2008). RhoU relocates to the plasma membrane upon serum stimulation in H1299 non-small cell lung cancer cells. The serum stimulation induces Src-mediated tyrosine phosphorylation of RhoU on Tyr254. This phosphorylation reduces GTPase activity and the ability of RhoU to interact with downstream effectors (Alan et al., 2010). In epithelial cells, RhoU was identified to bind to the scaffolding protein Par6 , which is well known for its role in regulating polarity downstream of Cdc42. This interaction was recognized affect epithelial cell tight junctions assembly and actin organization (Brady et al., 2009, Chen and Zhang, 2013). Much less has been reported for RhoV but it is known to bind to PAKs.

The interaction of RhoU with Nck2 could recruit these proteins indirectly and thereby promote cell migration (Blasutig et al., 2008). Nck SH2/SH3 adaptors act cooperatively to induce actin reorganisation; however, how RhoU proteins contribute to cancer progression mechanistically is still unknown. RhoV is abundant in cancer cell lines and others have shown that RhoV is up regulated in some human cancers (Aronheim et al., 1998). RhoV differs from RhoU at the C-terminus, which lacks a CAAX box and has a unique 32 amino acid sequence. Moreover, overexpression of RhoV can stimulate apoptosis and activation of Jun N-terminal kinase (JNK) signalling pathway via both death receptor-mediated and mitochondrial apoptotic pathways as determined by caspase-8 and caspase-9 activation, (Aronheim et al., 1998, Shepelev et al., 2011). This suggests a potential role of RhoV in regulating apoptosis in a JNK-dependent manner, but the association between RhoV and cancer progression remains to be determined.

Both RhoU and RhoV bind to some of the PAK family members, which are well known as effectors of Rac and Cdc42 (Aronheim et al., 1998, Tao et al., 2001). PAKs regulate cell migration and invasion and their overexpression is observed in some human tumours (Dummler et al., 2009). Deletion N-terminal of RhoU enhances its ability to bind PAK1 (Shutes et al., 2004). Overexpression of RhoV prompts downregulation of PAK1 and induces lamellipodia possibly through interaction with PAK2 (Aronheim et al., 1998, Weisz Hubsman et al., 2007). In MDA-MB231 cells, RhoU bind to PAK4. This interaction is kinase independent (Dart et al., 2015). In human embryonic kidney (HEK) cells, RhoV and RhoU bind to PAK6. Interaction between PAK6 and RhoV depends on the activation state of the GTPase, suggesting that PAK6 is an effector for RhoV. However, the interaction between PAK6 and RhoU were weaker (Shepelev and Korobko, 2012).

The ability of cancer cells to invade and metastasise is an important area of extensive research, and the identification of factors, proteins and signalling networks that contribute to this process is now required. One group of effector proteins downstream of RhoGTPases and contributing heavily to cell migration and metastasis are the family of p21-activated kinases (PAKs).

## **1.5. p21 activated kinase (PAK)**

### **1.5.1. PAKs overview**

The p21-activated kinases (PAKs) are serine/threonine kinases initially identified in screens for Cdc42 and Rac effectors, and there are six PAK isoforms (Manser et al., 1994, King et al., 2014). These kinases are highly conserved across a wide range of organisms including flies, yeast and humans (Bokoch, 2003). Human PAKs subdivide into two groups; based on their structural similarities, sequence homology and regulation. Group I PAKs include PAK1-3, and group II PAKs include PAK4-6 (Arias-Romero and Chernoff, 2008, Bokoch, 2003).

It is well known that PAKs act as effectors for a wide range of different signalling pathways. Activated PAKs can relocate to the nucleus, where they can manipulate gene transcription (Li et al., 2012, Tao et al., 2011, Sells and Chernoff, 1997), or alternatively, localise to the leading edge of cells to influence cytoskeletal organisation and thereby contribute to cellular motility (Arias-Romero and Chernoff, 2008, Bagrodia et al., 1999). Furthermore, it has been shown that PAKs can also function in hormone signalling (Lee et al., 2002, Schrantz et al., 2004, Rayala et al., 2006), changes in cell morphology (Cau et al., 2001, Manser et al., 1997, Zeng et al., 2000) and cell survival or apoptosis (Cotteret et al., 2003, Ong et al., 2011, Ye and Field, 2012).

Depletion of PAK genes in mice has different effects on viability and phenotype; depending on which PAK isoform has been silenced. In knockout studies, PAK1, PAK5 and PAK6 exhibited no obvious developmental requirement, as knockout mice are viable and fertile. However, PAK5 and PAK6 double knockout mice have deficits in locomotors activity as well as learning and memory but no reduction in their viability or fertility. (Arias-Romero and Chernoff, 2008, Nekrasova et al., 2008). In contrast, PAK2 and PAK4 knockout mice are embryonically lethal, which suggests essential roles for these two PAK isoforms during development (Arias-Romero and Chernoff, 2008, Qu et al., 2003). PAK3 knockout mice are viable but exhibit defects in synaptic plasticity, implicating crucial roles for PAK3 in neural differentiation (Qu et al., 2003).

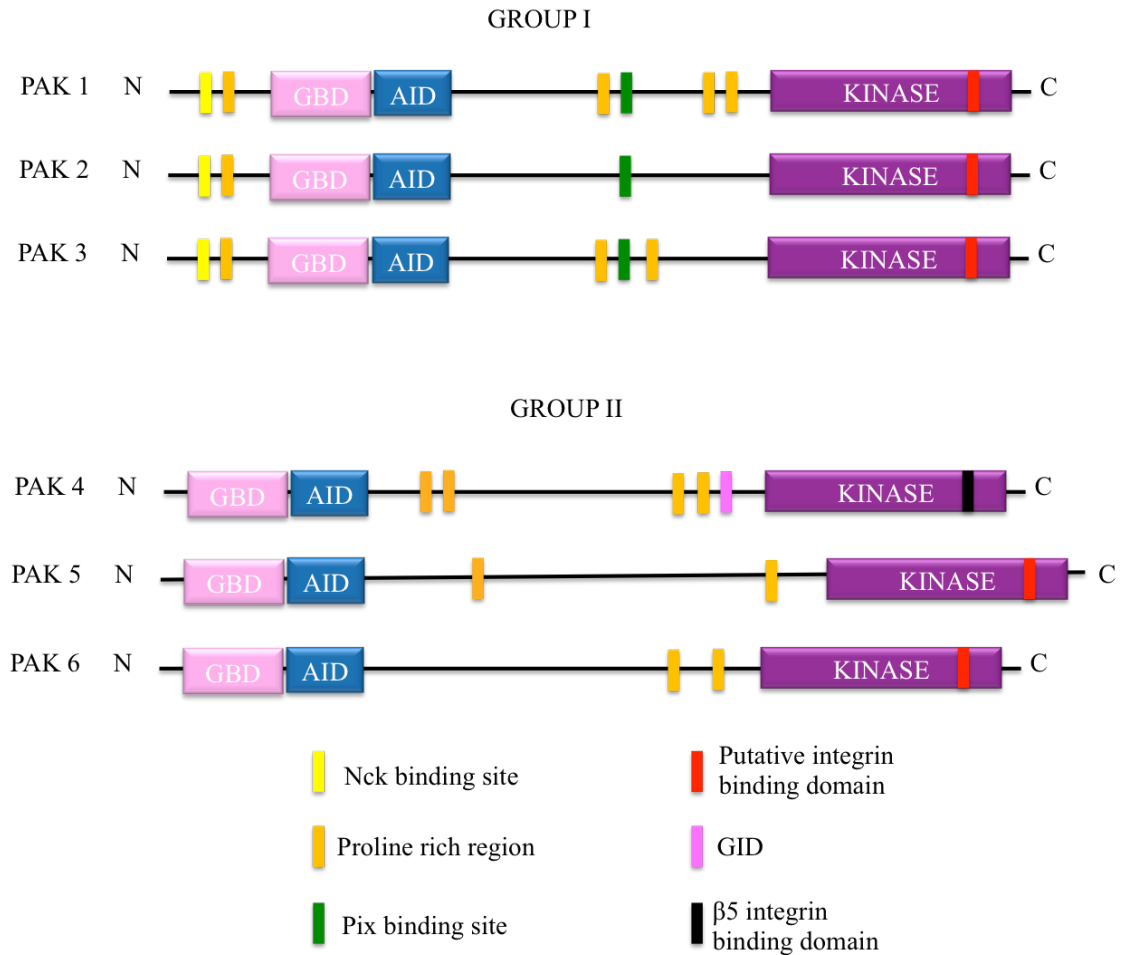
### **1.5.2. Domain architecture of PAKs**

PAKs exhibit significant sequence homology to the yeast Ste20 protein kinase (Bagrodia et al., 1999, Sells and Chernoff, 1997). Outside of the kinase domain, the sequences of PAKs and Ste20 protein kinase differ significantly except for a 60 amino acid sequence at the N-terminal, identified as the p21-GTPase binding domain (GBD) (Sells and Chernoff, 1997). All PAKs contain an N-terminal GBD as well as a highly conserved C-terminal catalytic serine/threonine kinase domain (Figure 1.8). Group I PAKs have a Cdc42/Rac interactive binding region (CRIB) within the GBD, which overlaps with an autoinhibitory domain (AID) (Arias-Romero and Chernoff, 2008, Eswaran et al., 2007, Whale, 2011). In contrast, group II PAKs were not thought to contain an AID regionally, but more recently a putative AID has been identified in PAK4 (Baskaran et al., 2012), and this region is conserved across in all group II PAKs (Dart and Wells, 2013). Moreover, all PAK proteins contain a different number of proline motifs (PxxP), which are putative binding sites for the SH3 domain containing proteins (Jaffer and Chernoff, 2002). The SH3 domain located at the extreme N-terminal region of PAK1 promotes the binding of the adaptor protein Nck (Bokoch et al., 1996) and growth factor receptor bound protein 2 (Grb2) (Puto et al., 2003). In addition, PAK1 interacts with the PAK-interacting exchange factor (PIX) (Manser et al., 1998), which leads to the translocation of a PAK1-Nck complex to the plasma membrane upon receptor activation (Lu et al., 1997). The N-terminal sequence to the GBD is smaller in group II PAKs compared to that in group I PAKs. PAK5 has been shown to possess an inhibitory region that is approximately 120 amino acids in size (Ching et al., 2003). It is reported that in PAK4, there is a GEF-H1/Gab1- interacting domain (GID), which binds to PDZ-RhoGEF (Barac et al., 2004a, Callow et al., 2005a). PAK4 has also been shown to interact with the integrin  $\alpha\text{v}\beta 5$  via a binding site located within the kinase domain (Li et al., 2010, Zhang, 2002).

The kinase activity of PAK4 is critical, PAK4 mediated phosphorylation of two serine residues in the integrin  $\beta 5$  cytoplasmic domain (Li et al., 2010). These phosphorylation events help to accelerate integrin  $\alpha \nu \beta 5$  turnover within adhesions through inhibition of integrin  $\alpha \nu \beta 5$  clustering, connections to F-actin and preventing adhesion maturation (Li et al., 2010), which is dependent on PAK4 kinase activity and promotes cell motility.

Although there are conserved structural regions within the PAK family of proteins, all of the isoforms are structurally different, suggesting that these sequences could provide different functional roles, substrate specificity and also allow recognition of specific target sequences on binding partners (Arias-Romero and Chernoff, 2008, Rennefahrt et al., 2007, Wells and Jones, 2010). However, despite these structural variances, PAK4 and PAK1 can also share a number of substrates as shown in Table 1.1.

PAK6 kinase is unique among PAKs in that this protein possesses an FXXMF motif, which links directly to the androgen receptor (AR) ligand-binding domain (LBD) (van de Wijngaart et al., 2006). Although PAK6 is similar to PAK1 and binds to the alpha isoform of the estrogen receptor (ER) (Lee et al., 2002, Wang et al., 2002), PAK6 is the only PAK family member shown to interact with the AR (Yang et al., 2001). Similarities and differences between the structures of group I and II PAKs are illustrated in Figure 1.8.



**Figure 1.8: PAK family member and structures.** There are six members of PAK family kinases, which are divided into two groups according to structural similarities and sequence homology. All PAKs contain a variable number of proline rich regions, which are putative binding sites for SH3 domain containing proteins. Group I PAKs also contains PIX binding site in the central region and Nck binding site at the N-terminal (indicated in green and yellow, respectively). Specific to PAK4, a  $\beta$ 5-integrin binding site (although this region is highly homologous among the other PAK family members) and a GEF-H1/Gab1 interacting domain (GID), which lies adjacent to the kinase domain. Figure adapted from (King et al., 2014).

**Table 1.1:** PAK substrates.

<b>Substrate</b>	<b>Cellular function</b>	<b>PAK</b>	<b>Reference</b>
Caldesmon	Inhibitor of myosin ATPase activity	PAK1 & 3	(Foster et al., 2000)
CPI17	Inhibitor of myosin phosphatase	PAK1	(Takizawa et al., 2002)
Desmin	Intermediate filament protein	PAK1	(Ohtakara et al., 2000)
FilaminA	Actin cross linking and adhesion protein	PAK1	(Vadlamudi et al., 2002a)
GIT1	GTPase regulation Arf GAP	PAK1	(Zhao et al., 2005)
GEF-H1	RhoGTPase regulation, RhoA GEF	PAK1 & 4	(Zenke et al., 2004a) (Callow et al., 2005b)
LIMK1	Actin cytoskeleton dynamics; cofilin kinase	PAK1, 2 & 4	(Dan et al., 2001a) (Edwards et al., 1999)
MLCK	Regulation of myosin activity and actin cytoskeleton dynamics	PAK1 & 2	(Sanders et al., 1999) (Goeckeler et al., 2000)
Merlin	ERM binding protein	PAK2	(Kissil et al., 2002)
p41-ARC	Subunit of Arp2/3 complex, actin nucleation	PAK1	(Vadlamudi et al., 2002a)
Paxillin	Focal adhesion scaffold	PAK4	(Wells et al., 2010b)
PDZ-RhoGEF	RhoGTPase regulation, RhoA GEF	PAK4	(Barac et al., 2004b)
PIX	RhoGTPase regulation, Rac GEF	PAK1 & 2	(Shin et al., 2002, ten Klooster et al., 2006)
Raf-1	MEK kinase	PAK1,2, 3&4	(King et al., 1998, Zang et al., 2002)
Rho-GDI	Inhibitor of RhoGTPase activity	PAK1	(DerMardirossian et al., 2004)
R-MLC	Regulatory chain of myosin motor	PAK2	(Chew et al., 1998)
SSH-1	Actin cytoskeleton dynamics; cofilin phosphatase	PAK4	(Soosairajah et al., 2005)
Vimentin	Intermediate filament protein	PAK1	(Goto et al., 2002)



### 1.5.3. Regulation of PAK activity

Group I PAKs have a low basal level of kinase activity, which is enhanced by the binding of Cdc42 or Rac1 to the GBD. In the inactive state, the group I PAKs form unphosphorylated homodimers whereby the AID binds to the kinase domain and prevents its activity. Active Cdc42 or Rac1 binding to GBD of group I PAKs disrupts dimerisation, resulting in autophosphorylation at several serine residues and single threonine residue (corresponding to Thr<sup>423</sup> in PAK1 and Thr<sup>402</sup> in PAK2), located within the activation loop of the kinase domain which results in kinase activation (Zenke et al., 1999, Arias-Romero and Chernoff, 2008, Eswaran et al., 2008, Molli et al., 2009). Distinctively, the PAK3 gene has splice exons that have been detected in the GBD/AID region, resulting in four splice variants (Kreis et al., 2008). These splice variants have not been identified in PAK1/2. The change in GBD/AID structure of three PAK3 variants results in increase the kinase activity and modifies the GTPase binding. The expression of the new PAK3 (PAK3b, PAK3c, and PAK3cb) splice variants in transfected COS7 cells, induces morphological changes of cell shape and leads to modifications of focal adhesion structure (Kreis et al., 2008).

In contrast to group I PAKs, the binding of active Cdc42 has no significant impact on the kinase domain of group II PAKs. It is reported that PAK4 has a high basal kinase activity that is not affected by the binding of the activated small GTPases, Cdc42 and Rac1 (Abo et al., 1998). With the exception of PAK5, it was originally thought that group II PAKs had no AID present and there has been much speculation over the regulation of PAK4. Recently, however, studies have suggested two possible mechanisms of PAK4 activity regulation. The first mechanism identifies a potential AID located in the N-terminal domain that is composed of amino acids 20-68, which is thought to maintain PAK4 in an inactive conformation until the binding of active Cdc42 leads to structural changes causing PAK4 activation (Baskaran et al., 2012). In another study, researchers proposed that the PAK4 regulatory domain possesses an N-terminal autoinhibitory pseudosubstrate motif, which binds the kinase domain (Ha et al., 2012). These reports suggest that PAK4 activity is controlled by the N-terminal AID and not by the autophosphorylation of PAK4 at Ser474, which is now suggested to be constitutively phosphorylated at this site. Moreover, as this region is highly conserved in all group II PAKs it is likely that this process of regulation is also exhibited by PAK5 and

## PAK6.

The regulation of PAK kinase activity not only occurs through the interaction of Cdc42 and Rac1 GTPases but also via a variety of different proteins and pathways. PAKs activated by extracellular signals can contribute to several signalling networks. PAKs can activate mitogen-activated protein kinase (Menges et al., 2012) as well as phosphorylating a number of regulators of the cytoskeleton, such as LIM domain kinase (LIMK), PAK1 can activate LIMK1 (Edwards et al., 1999), and PAK4 can bind to and phosphorylate LIMK1, which stimulates phosphorylation of cofilin (Dan et al., 2001b). Furthermore, the phosphorylation of the pro-apoptotic proteins BAD and dynein light chain 1 (DLC1) provide another pathway that PAK1 could stimulate to enhance breast cancer cell survival. A number of studies have demonstrated that PAK1 and/or PAK2 can directly phosphorylate myosin light-chain (MLC), thereby increasing contractility (Zeng et al., 2000).

The interaction between PAK6 and active Cdc42 does not enhance PAK6 kinase activity (Schrantz et al., 2004), but it is likely that this association modulates PAK6 localisation as observed for PAK4 (Abo et al., 1998). PAK6 interacts with Rac1 (Civiero et al., 2015) and the atypical RhoGTPase family member, RhoV, which has 52% sequence homology to Cdc42, and RhoV has been implicated in the modulation of cytoskeletal dynamics (Aronheim et al., 1998, Shepelev and Korobko, 2012). It has been reported that full-length PAK6 exhibits reduced kinase activity when compared to a truncated version of PAK6 containing only the protein kinase domain (Yang et al., 2001). This suggests that PAK6 may also be autoinhibited. PAK6 kinase activity can also be regulated by MAPK signalling cascade. Phosphorylation by MAPK at serine 165 activated protein kinase kinase 6 (MKK6) activates PAK6 (Kaur et al., 2005). Moreover, autophosphorylation of PAK6 at serine 560 is required for MKK6-facilitated activation (Kaur et al., 2005). Furthermore, the results from kinase activity studies suggest that the AR may also have a role in regulating PAK6 kinase activity in prostate cancer cells (Lee et al., 2002).

#### 1.5.4. PAK expression in cancer

Given that PAKs are involved in many cellular activities including proliferation, cell cycle regulation and apoptosis, it is likely that this family of kinases may also have roles in regulating cancer growth and progression. The overexpression of group I PAKs is detected in a wide variety of different cancers. PAK1 is most commonly overexpressed (King et al., 2014), and increased levels of PAK1 have been detected in human cancer tissue from breast (Ong et al., 2011, Holm et al., 2006), lung (Ong et al., 2011), liver (Ching et al., 2007) and melanoma (Ong et al., 2013). PAK2 overexpression has been observed in breast (Li et al., 2011) and lung cancer tissue (Kichina et al., 2010). PAK3 has been found to be present in neuroendocrine tumours (Liu et al., 2010).

Within Group II PAKs, PAK4 is considered to have a ubiquitous expression, with particularly elevated levels of PAK4 detected in a number of cancer cell lines and tumour type including prostate, breast, and pancreatic (Callow et al., 2002, Kumar et al., 2006). PAK5 is found to be expressed predominantly in the brain, where it promotes neurite outgrowth (Dan et al., 2002, Pandey et al., 2002), but it is also expressed in colorectal cancers (Gong et al., 2009). PAK6 is expressed in prostate and breast cancer cell lines (Kaur et al., 2008b). Moreover, PAK6 expression is elevated in prostate cancer tissue and colon cancer tissue in comparison to benign samples. Furthermore, PAK6 was overexpressed in hepatocellular carcinoma (HCC) (Kaur et al., 2008b, Zhang et al., 2010, Chen et al., 2014). In contrast, in clear cell renal cell carcinoma, the intratumoral PAK6 expression was significantly lower than that in nontumoral tissues (Liu et al., 2014).

Generally, much of the attention has been directed towards founding family member PAK1. However, the focus is currently shifting towards group II PAK. PAK4 is a pluripotent kinase regulating several biological processes ranging from cell motility to survival. To date, it is the best characterised of the Group II PAKs with more various roles for this kinase rapidly emerging every year. Moreover, there is a significant work linking PAK4 to the critical control of the cytoskeleton. As more binding partners and substrates are discovered for group II PAK, It will be interesting to learn whether differences in upstream regulators and/or spatial control of subcellular localization contribute to PAK isoform. Furthermore, the functional importance of these interactions *in vivo*, especially some of them shared substrate specificity with PAK1. Although small molecule inhibitors of PAK4 have yet to prove

successful, the continued understanding of group II PAK biology will be a valuable resource in strengthening the basis for new therapeutic interventions. The future of group II research is an exciting prospect as we expand our knowledge of the roles of this kinase and validate its effectiveness as a drug target.

#### **1.5.5. Group II PAKs: Cancer cell metastasis**

PAK proteins have been strongly associated with cytoskeletal dynamics via their regulation of actin assembly and consequently are thought to contribute to cell migration (Bokoch, 2003, Dharmawardhane et al., 1997, Jaffer and Chernoff, 2002). Whilst there is increasing evidence of a strong link between PAK4 and cancer cell migration, PAK4 was initially identified as a cytoskeletal regulatory kinase, acting in response to a direct interaction with Cdc42 (Abo et al., 1998). One study demonstrated that PAK4 interacts with and phosphorylates the GEF, GEF-H1 and this activity may permit PAK4 to interact with microtubules, which subsequently affects cellular morphology and motility (Callow et al., 2005a). Cofilin is a target of LIMK and a key regulator of actin filament disassembly. PAK4 also interacts with and phosphorylates LIMK, increasing its ability to phosphorylate cofilin and thereby inhibiting actin disassembly (Dan et al., 2001b). This interaction between PAK4 and LIMK has been shown to occur downstream of HGF in prostate cancer cells (Ahmed et al., 2008). Moreover, in response to HGF, PAK4 associates with the scaffold protein Gab1 that drives localisation to the cell periphery, specifically within lamellipodia (Paliouras et al., 2009), and enhance cell migration (Ahmed et al., 2008). In contrast, prostate cancer cells lacking PAK4 are less responsive to HGF stimulation with the significantly reduced level of cell migration observed (Wells et al., 2010a). In addition, constitutively active PAK4 increases the invasiveness of pancreatic ductal cells, whereas the knockdown of PAK4 decreases growth and reduces the migratory capacity of a highly invasive pancreatic cell line (Kimmelman et al., 2008).

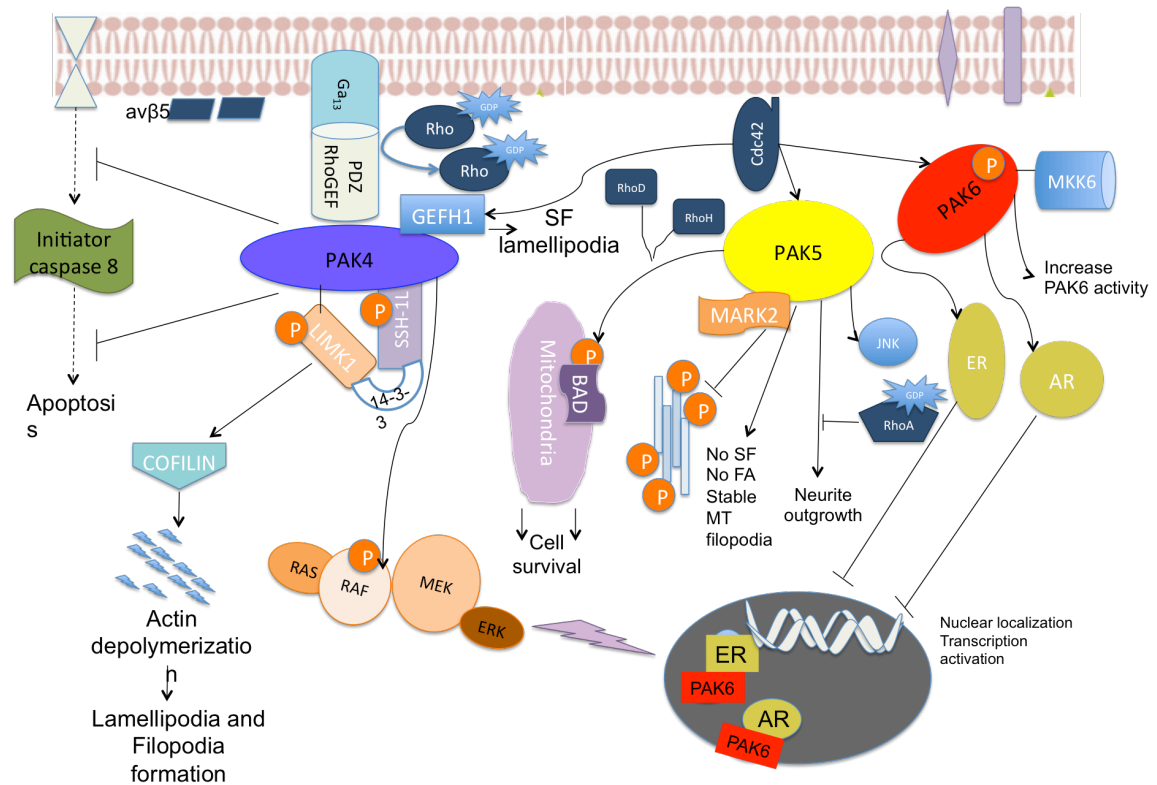
Others have shown that PAK5, downstream of active Rac/Cdc42, colocalises and binds to both actin and microtubule structures in *Xenopus*. Within the *Xenopus* model, X-PAK5 is expressed early on during development, particularly within the regions where morphogenetic movements are induced during the onset of gastrulation (Cau et al., 2001, Faure et al., 2005).

All these studies indicate a role of PAK5 in cell migration. In addition, PAK5 has been linked to filopodial formation during neurite outgrowth. The overexpression of PAK5 in N1E-115 neuroblastoma cells induces outgrowth as well as filopodia formation (Dan et al., 2002). Moreover, overexpressed PAK5 in colorectal carcinoma cell lines localises to focal adhesions and the leading edge (Gong et al., 2009) and can reduce adhesion and increase the migratory response on collagen I (Gong et al., 2009). These studies on PAK5 are similar to the reported PAK4 functions in cell adhesion and could suggest an overlap between these proteins.

It has been shown that the PAK5-Egr1-MMP2 signalling pathway could be crucial in breast cancer cell migration and invasion in both BT549 and MDA-MB-231 cells (Wang et al., 2013). Here silencing PAK5 reduced breast cancer cell line proliferation, promoted apoptosis, and decreased cell migration in wound-healing and matrigel transwell invasion assay. Depletion of PAK5 expression in BT549 and MDA-MB-231 cells was associated with a decreased level of cleaved MMP2 and increased level of Egr1 expression. Egr1 is a transcription factor which can bind to the MMP2 promoter and inhibit its activity (Zcharia et al., 2012), and MMP2 is one of the proteases involved in breast cancer invasion, associated with poor prognosis (Talvensaari-Mattila et al., 2001, Talvensaari-Mattila et al., 2003). These results suggest a mechanism through which PAK5 could play a significant role in cancer cell migration and invasion.

In contrast to the other Group II PAKs, there is much less known about the interaction between PAK6 and changes in cytoskeletal dynamics. However, it has been reported that PAK6 interacts with IQGAP1 and protein phosphatase 1B in breast cancer cells (Kaur et al., 2008b). This binding was confirmed by our group in prostate cancer cells demonstrating that PAK6 and IQGAP1 can function to induce cell:cell dissociation downstream of HGF in DU145 cells. Furthermore, it has been shown that E-cadherin, IQGAP1 and PAK6 are present in a protein complex (Fram et al., 2014). While group I and II PAKs interact with the same Rho GTPases, the interaction with specific Rho family members might direct the

specific downstream signalling of individual PAK isoforms (Figure 1.9). Although an overview of all PAK members has been provided, PAK6 serves as the main focus of the work described in this thesis.



**Figure 1.9: Group II PAK mediated signalling pathways.** The group II PAKs, PAK4 (purple), PAK5 (yellow) and PAK6 (red), mediate signalling cascades that influence cytoskeletal reorganization, neuronal development, cell survival and hormone signalling. PAK-mediated substrate phosphorylation is shown using the letter P. Abbreviations: AR, androgen receptor; BAD, Bcl-2 agonist of cell death; ER, oestrogen receptor; FA, focal adhesion; JNK, c-Jun N-terminal kinase; MKK, MAP kinase kinase 6; MT, microtubule; PDZ, post-synaptic density protein (PSD95), Drosophila disc large tumour suppressor (DlgA), and zonula occludins-1 protein (zo-1); SF, stress fibres. Adopted from (Eswaran et al., 2008).

### 1.5.6. PAK6

PAK6 was detected in a screen to identify proteins that interact with the androgen receptor (AR) (Yang et al., 2001). PAK6 binds to the ligand-binding domain (LBD) of the AR and triggers suppression of AR signalling. The ability of PAK6 to bind to steroid hormone receptors suggest that it could contribute to the hormonal independence that is representative of many aggressive tumours (Schrantz et al., 2004). In support of this notion, PAK6 expression is increased in both prostate and breast cancer cell lines (Kaur et al., 2008b). Reduced PAK6 expression combined with irradiation can decrease the survival of prostate cancer cells (Zhang et al., 2010) and PAK6 may protect these cells from apoptosis by phosphorylating the proapoptotic protein BAD on Ser<sup>112</sup> (Zhang et al., 2010). Consistent with this observation, a combination of PAK6 inhibition and 5-fluorouracil (5-FU) treatment causes significantly decreased survival of colon cancer cells, and this result has been reproduced *in vivo* (Chen et al., 2014).

In prostate cancer, cell growth was inhibited upon PAK6 knockdown and this effect was enhanced in the presence of the anti-tumour compound, docetaxel (Wen et al., 2009). Furthermore, the depletion of PAK6 in prostate cancer cells resulted in impaired PC3 cell migration and reduced expression of matrix metalloproteinase 9 (Goc et al., 2013). Notably, in some prostate cancer cells, the PAK6 gene is hypermethylated, and this would ordinarily lead to downregulation of expression.

Our group found that knockdown of PAK6 expression in DU145 prostate cancer cells inhibited HGF induced cell:cell junction dissociation and cell scattering. In contrast, overexpression of PAK6 caused cell dissemination (Fram et al., 2014). It is known that PAK6 shows abundant localisation in the mitochondria (Cotteret et al., 2003). In addition, PAK6 has been detected in the cytoplasm and at the plasma membrane of HeLa cells (Lee et al., 2002), as well as in the nucleus of prostate cells (Yang et al., 2001). Overexpressed PAK6 can also localise to punctate cytoplasmic structures in HeLa and NCI-H1299 lung cancer cells (Shepelev and Korobko, 2012). Despite these investigations of PAK6 function in prostate cancer, the involvement of PAK6 in the development of breast cancer has not been studied previously.



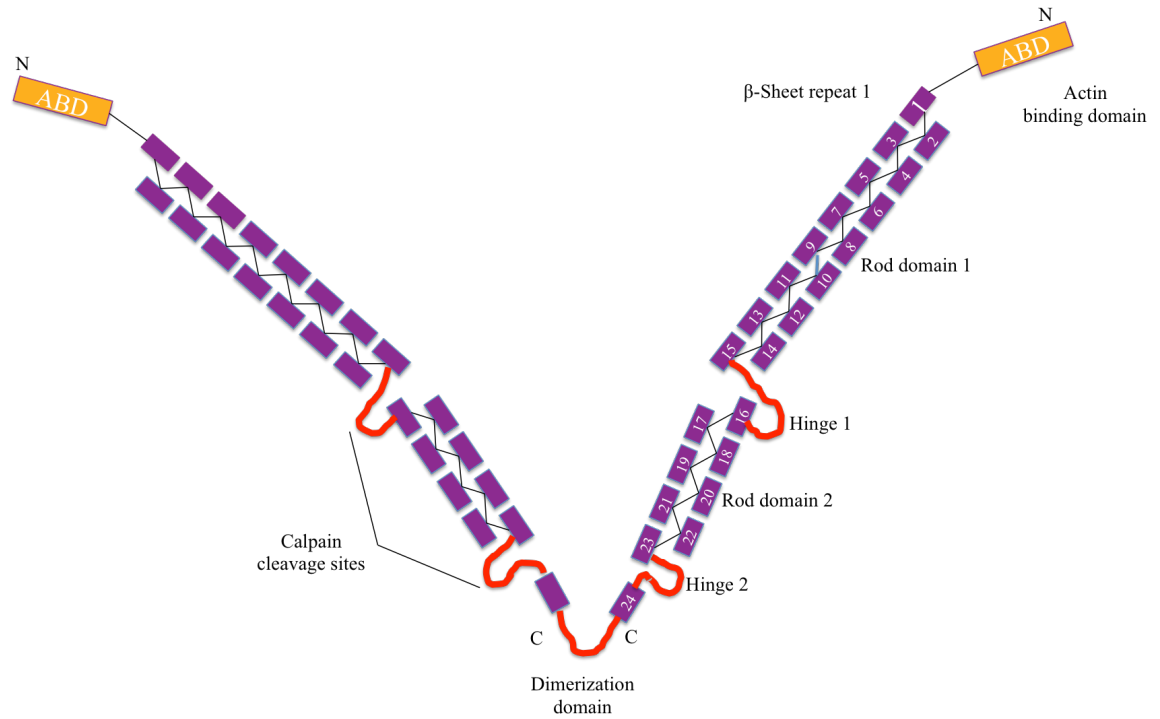
## 1.6. FilaminA

Of particular interest in this thesis is FilaminA, which has been identified as a novel PAK6 binding protein that has also been previously linked to the PAK family. The up-regulation of phosphorylated PAK1 leads to increased breast cancer cell motility by FilaminA (Hammer et al., 2013). In addition, the interaction between PAK1 and FilaminA could contribute to the stimulation of PAK1 activity and its targets in cytoskeletal structures (Vadlamudi et al., 2002b). Clinical studies have shown that FilaminA is overexpressed in metastatic breast cancer (Jiang et al., 2013, Alper et al., 2009, Tian et al., 2013). It is reported that the overexpression of cytoplasmic FilaminA is linked with advanced stage, lymph node metastasis and vascular or neural invasion of breast cancer (Tian et al., 2013).

In addition, low levels of FilaminA expression are associated with a better distant metastasis-free survival compared to patients with normal levels of FilaminA (Jiang et al., 2013). The circulating levels of FilaminA in patient's plasma are higher in cases with metastatic breast cancer (Alper et al., 2009).

FilaminA is also referred to as actin-binding protein 280 (ABP 280), and this protein was first identified as a non-muscle actin filament cross-linking protein. It is a cytoskeletal protein that cross-links F-actin into either networks or stress fibres, (Niedeman, 1983, Hartwig and Stossel, 1975). The name Filamin refers to its filamentous colocalisation with actin stress fibres (Wang et al., 1975). In mammals, there are three highly homologous filamins; Filamins A, B and C. The full-length FilaminA consists of an N-terminal, which contains an actin-binding domain (ABD), and a C-terminal end followed by 24 immunoglobulin (Ig)-like repeat domains, which are folded into  $\beta$ -sheets and together, form a rod structure as illustrated in Figure 1.10 (Ruskamo et al., 2012). The ABD of FilaminA contains two-calponin homology domain (CH1 and CH2). Calponin homology domains have been shown to facilitate the actin-binding properties of multiple proteins, including dystrophin, utrophin,  $\alpha$ -actinin and  $\beta$ -spectrin (Gimona et al., 2002, Ruskamo and Ylanne, 2009). FilaminA cross-link F-actin through ABD, and therefore interact with a multiplicity of other transmembrane and peripheral membrane proteins. Furthermore, FilaminA also contains nuclear localisation signals (NLS) in the ABD in repeat 14 and repeat 20. Two

hinge regions are located between repeats 15 and 16 and between repeats 23 and 24, which allow the protein to bend when, the molecules homodimerise through repeat 24. This occurs when the protein dimerises and the structure is similar to a Y, with dimerization occurring at the 24th repeat (van der Flier and Sonnenberg, 2001, Robertson, 2005). Dimerisation permits FilaminA to control the structure of the cell through actin binding in multiple ways.



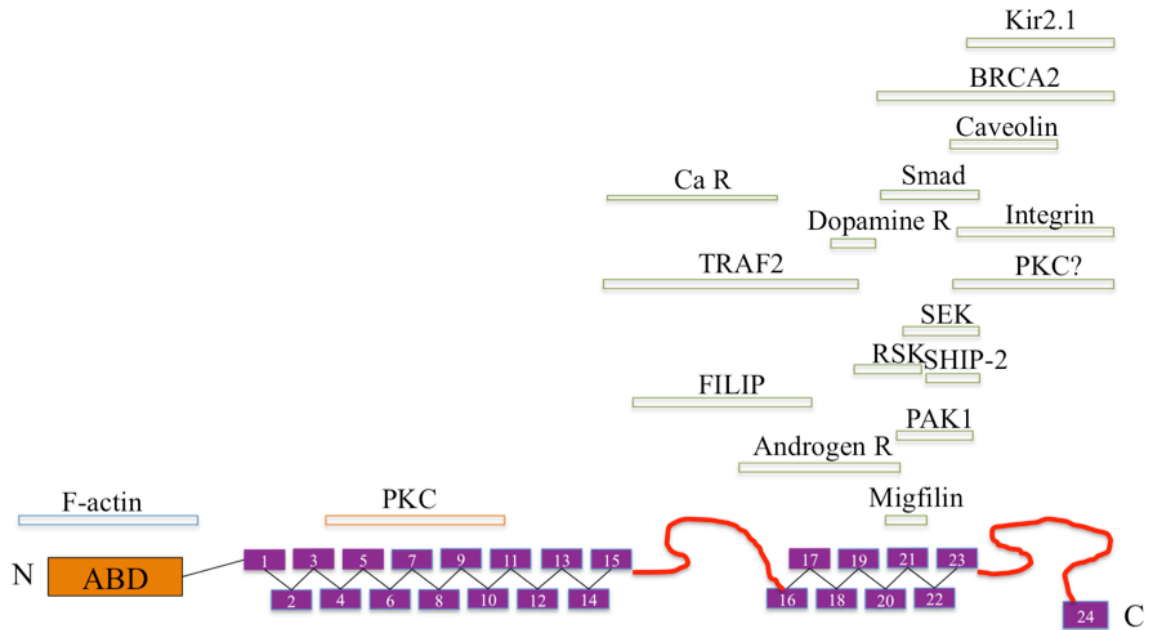
**Figure 1.10: The structure of FilaminA.** The amino-terminal actin-binding domain is represented in orange and the chain of 24 filamin repeats in purple. Two hinge regions are located between repeats 15 and 16 and between repeats 23 and 24. Dimerization happens through the twenty-fourth repeat. Figure is adapted from (Shao et al., 2015).

### **1.6.1. Biological function of Filamins**

FilaminA function is regulated at many levels by phosphorylation, proteolysis, the binding of phospholipids, as well as organellar compartmentalisation (Gorlin J, 1990, Liu et al., 1997, Woo et al., 2004). Filamins act as scaffolding molecules, facilitating protein–protein interactions and influencing protein cellular localisation. Over 90 binding partners, including receptors, intracellular signalling molecules, and transcription factors have been identified (Nakamura et al., 2011). FilaminA binds to functionally diverse proteins through repeats 14–24, as shown in Figure 1.11. It is reported that FilaminA can bind to the Rac-specific GTPase binding protein FilGAP, and FilGAP localises to sites of membrane protrusion where it is involved in lamellipodia formation. Moreover, cell spreading is disturbed by this interaction through mutations at the FilGAP binding site in Filamin (Nakamura et al., 2009, Ohta et al., 2006b, MacPherson and Fagerholm, 2010). Subsequent reports revealed the importance of this protein in the cytoplasm, cell contraction, and cell spreading (Hartwig and Stossel, 1975). Therefore, these results have highlighted the importance of FilaminA in regulating cell migration (Price et al., 1994, de Wit et al., 2009). FilaminA is necessary in cell migration to recruit proteins to the sites of migration, such as filopodia and lamellipodia, and these proteins can then function to the cell surface to allow cell movement (Nakamura et al., 2011). On the other hand, overexpression of wild type FilaminA can inhibit neuronal migration, thereby demonstrating that optimum levels of FilaminA are required for appropriate development (Sarkisian et al. 2006).

The exact role of Filamin during migration and whether its mechanosensing activities are essential remains unclear. It has been shown that cells deficient in Filamins are impaired in initiation of migration but are nevertheless capable of migrating at normal speeds (Baldassarre et al., 2009). This finding suggests that Filamins are not essential for migration processes but they could contribute to its regulation, possibly through the formation of mechanosensitive associates between integrins and the actin cytoskeleton (Stossel et al., 2001).

Mutations in the FilaminA gene are detected in a wide range of disease phenotypes, involving, cardiovascular malformations, terminal osseous dysplasia, and neural defects (Robertson, 2005, Nakamura et al., 2011, Stossel et al., 2001). It is reported that the loss of FilaminA is also embryonically lethal (Feng et al., 2006). Furthermore, the lack of FilaminA results in male lethality due to incomplete separation of the outflow tract of the heart, which produces common arterial trunk and midline fusion, defects manifesting as sternum and palate abnormalities (Hart et al., 2006).



**Figure 1.11: Diagram illustrating Filamin interactions.** The ABD at the N-terminal of filamin includes two-calponin homology domain. The 24 repeats that follow the ABD most probably fold into antiparallel  $\beta$ -sheets and function as interfaces for protein-protein interaction. These repeats not only mediate the dimerization of filamin through C- terminal repeat 24, but also allow filamin to interact with different proteins. Figure is adapted from (Feng and Walsh, 2004).

### **1.6.2. FilaminA in Cancer Invasion and Metastasis**

FilaminA is expressed in many human cancers. It can act as a cancer-promoting protein and is likely to be of fundamental importance in cell migration and metastasis (Zhou et al., 2011). However, recent reports have also found that it prevents tumour progression, indicating that FilaminA could have a dual role in cancer (Savoy and Ghosh, 2013b). FilaminA may likely play a role in cancer metastasis due to the fact of its involvement in multiple associated regulatory pathways (Zhou et al., 2010). The association between R-Ras and FilaminA can significantly enhance melanoma migration (Gawecka et al., 2010). Moreover, the inhibition of FilaminA significantly reduces the migration and invasion of cancer cells (Jiang et al., 2013). Targeting FilaminA through depletion experiments has helped investigators understand the role of FilaminA in cancer progress and its importance in cancer cell growth and metastasis (Savoy and Ghosh, 2013a). Many studies have indicated that although full-length FilaminA is generally localised to the cytoplasm, or plasma membrane, it has a tumour-promoting effect due to its interaction with signalling molecules (Savoy and Ghosh, 2013b). On the other hand, if FilaminA is localised to the nucleus, then it may act to suppress tumour growth and inhibit metastasis by interacting with transcription factors (Ozanne et al., 2000, Loy et al., 2003, Bedolla et al., 2009, Wang et al., 2007). It is reported that in the cytoplasm, full-length FilaminA stimulates the development of metastasis. This hypothesis is based on immunohistochemistry studies of a human prostate tissue microarray; they found that FilaminA proteolysis is associated with a decrease of metastatic potential of prostate cancer. Prostate cancer metastasis correlates with cytoplasmic localization of full-length FilaminA but not nuclear FilaminA fragments (Yue et al., 2013, Bedolla et al., 2009). It was also suggested that metastasis may be prevented by cleavage and subsequent nuclear translocation of this protein, where nuclear FilaminA was exhibited to be essential for inhibition of transcription and susceptibility to therapeutic interventions (Loy et al., 2003, Wang et al., 2007).

FilaminA function in the cell depends on the binding partners and acts to bring proteins together and promote the interaction of these proteins to either promote or prevent cancer (Savoy and Ghosh, 2013a). Pre-clinical studies show that FilaminA has a dual role in breast cancer metastasis. In MCF-7 human breast cancer cells stably expressing caveolin-1 (MCF-7/Cav1cells), FilaminA expression is upregulated and is phosphorylated on Ser-2152 in response to IGF-I stimulation, likely mediated by the PI3K/Akt pathway. It was further demonstrated that MCF-7/Cav1 cells exhibit substantially higher migration rates when compared with control MCF-7 cells (Xu et al., 2010, Ravid et al., 2008, Ravid et al., 2005). Cyclin D1 is also known to bind to FilaminA, furthermore the level of two phosphorylated forms of FilaminA S2152 and S1459 is reduced in cyclin D1 siRNA treated cells (Zhong et al., 2010, Jiang et al., 2013, Tian et al., 2013). Interestingly, cyclin D1 knockdown and inhibition resulted in decreased motility and an impaired wound healing response in MDA-MB-231 breast cancer cell lines, which maybe correlated with the changes in FilaminA phosphorylation (Zhong et al., 2010).



### **1.7. Aims of the project**

The aim was to characterise the role of PAK6 in breast cancer cells and to investigate PAK6 binding partners that may affect cell migration. More specifically:

- Characterise the expression of PAK6 in a panel of breast cancer cell lines and to investigate the effect on cellular behaviour of PAK6 overexpression in breast cancer cells
- Investigate the effect of PAK6 specific knockdown on cell morphology and associated pathways.
- Identify breast cancer relevant PAK6 binding partners by immunoprecipitation and mass spectrometry and to characterise the relationship between PAK6 and identified interacting partners.

## **Chapter 2:**

# **Materials and Methods**

## 2. Chapter 2: Materials and Methods

### 2.1. Materials

#### 2.1.1. General Materials

**Table 2.1:** General Materials.

Reagents	Source
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)	GIBCO®, Invitrogen, UK
4', 6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, UK
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	Sigma- Aldrich, UK
96-well, U-bottomed, suspension culture plate	Greiner, UK
Acetic acid	Sigma-Aldrich, UK
Agarose	Invitrogen. UK
Ammonium persulfate (APS)	Sigma-Aldrich, UK
Acrylamide (30%)	Severn Biotech Ltd, UK
Alexa Fluor 488 Goat anti Mouse antibody	Invitrogen, UK
Ampicillin	Sigma Aldrich. UK
Aprotinin	Sigma-Aldrich, UK
Beta- mercaptoethanol	Sigma-Aldrich, UK
Bromophenol Blue	Bio-Rad, UK
Bovine serum albumin (BSA)	(VWR International,UK)
Blebbistatin inhibitor	Calbiochem
Calcium phosphate transfection kit	Sigma-Aldrich, UK
Control siRNA oligonucleotide (non-silencing)	Qiagen Ltd, UK
Concentrated rat tail collagen, type I	BD Biosciences, UK
Coomassie brilliant blue	(Thermo Scientific, USA)
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen. UK
DMEM (1x) Powder, High Glucose	PAA, UK
DH5αTM Competent <i>Escherichia coli</i> ( <i>E. coli</i> ) Cells	Invitrogen, UK
Dithiothreitol (DTT)	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich. UK
Dulbecco's Phosphate buffered saline (PBS) without Calcium & Magnesium	PAA, UK
ECL enhance chemiluminescence Western Blotting Detection System	Thermo Scientific
EGF (Recombinant Human)	(R&D system, USA) GIBCO
Ethanol	(BDH Laboratory Supplies, U

Ethylenediaminetetraacetic acid (EDTA)	Gibco. UK
Flbronectin	Sigma-Aldrich. UK
Foetal Bovine Serum (FBS)	Gibco. UK
FluorSAVE reagent	Calbiochem, UK
HGF (Recombinant Human)	(R&D system, USA)
Isopropanol	(VWR International, UK)
Glycerol	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
HiPerfect transfection reagent	Qiagen Ltd, UK
Kanamycin	Invitrogen, UK
Leupeptin	Sigma-Aldrich. UK
Luria-Bertani agar (LB-agar)	Sigma-Aldrich, UK
Luria-Bertani broth (LB-broth)	Invitrogen. UK
Lipofectamine <sup>W</sup> 2000	Invitrogen. UK
Lithium chloride (LiCl)	Fisons Scientific Apparatus, U
Magnesium chloride (MgCl <sub>2</sub> )	Sigma-Aldrich, UK
Methylcellulose	Sigma-Aldrich,UK
Methanol	(VWR International, UK)
Non-fat milk powder	Marvel, UK
Nitrocellulose membrane	Perkin Elmer
Octylphenoxypolyethoxyethanol/Nonidet <sup>TM</sup> P40 substitute (NP-40)	Sigma-Aldrich,UK
PAK6 siRNA Oligo 1	Ambion
PAK6 siRNA Oligo 2	Thermo scientific
OptiMEM	GIBCO®, Invitrogen, UK
Panobinostat (LBH)	BioVision
Paraformaldehyde (PFA)	Sigma-Aldrich, UK
Penicillin & Streptomycin	Sigma-Aldrich, UK
Pierce® ECL western blotting substrate	Thermo Scientific, USA
Protein G Sepharose <sup>TM</sup> 4 Fast Flow beads	Amersham Biosciences, UK
Precision Plus Protein Marker	Bio-Rad, UK
Phosphate Buffered Saline (PBS) Tablets	Oxoid Ltd. UK
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich. UK
Fibronectin QIAGEN Plasmid maxi-prep kit	Qiagen Ltd, UK
RhoA Inhibitor	Cytoskeleton
ROCK Inhibitor	Calbiochem
Sodium chloride (NaCl)	Sigma-Aldrich, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, UK
Sodium fluoride (NaF)	Alfa Aesar, UK
Sodium hydroxide (NaOH)	Sigma-Aldrich, UK
Sodium orthovanadate (Na <sub>3</sub> VO <sub>4</sub> )	Sigma-Aldrich, UK

Sodium pyrophosphate	BDH Chemicals, UK
Tetramethylrhodamine isothiocyanate (TRITC)-Phalloidin	Sigma-Aldrich, UK
Tetramethylethylenediamine (TEMED)	(Sigma-Aldrich, UK)
Triton X-100	VWR International, UK
Tris Base	Sigma-Aldrich, UK
Trypsin/EDTA	GIBCO®, Invitrogen, UK
Tween 20	VWR International, UK
Whatman PROTRAN nitrocellulose membrane	(Perkin Elmer, USA)
X ray film	Scientific Laboratory Supplies

**Table 2.2:** Buffers and solutions.

<b>Buffer</b>	<b>Composition</b>
Blocking solution	5% w/v milk powder or 5% w/v bovine serum albumin in TBS-Tween
Coomassie blue stain	50% v/v methanol, 10% v/v acetic acid, 0.025% w/v coomassie blue
Collagen I matrix	1.617mg/mL Purecol collagen type I, 3mM NaOH, 10% FBS in DMEM
GFP Trap Lysis buffer	10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40
GFP-TRAP wash buffer	10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA
NP-40 lysis buffer	0.5% NP-40, 30mM sodium pyrophosphate ( $\text{Na}_{407}\text{P}_2$ ), 50mM Tris-HCl pH7.6, 150mM NaCl, 0.1mM EDTA plus protease inhibitor cocktail
PBS-Tween	PBS + 0.1% Tween-20
Protease inhibitor cocktail	50mM NaF, 1mM $\text{Na}_3\text{VO}_4$ , 1mM PMSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ aprotinin and 1mM DTT
Pulldown lysis buffer	20mM Tris-HCl pH7.6, 150mM NaCl, 2mM EDTA, 0.01% v/v TritonX-100, 10% v/v glycerol plus protease inhibitor cocktail
Pulldown wash buffer	20mM Tris-HCl pH7.6, 300mM NaCl, 2mM EDTA, 1% v/v TritonX-100, 10% v/v glycerol plus protease inhibitor cocktail
Laemmli buffer (6X)	375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% $\beta$ mercaptoethanol, 0.03% bromophenol blue
SDS-PAGE running buffer (10X):	250mM Tris-base, 1.92M Glycine, 1% w/v SDS. Dilute to 1X with distilled water
SDS-PAGE transfer	250mM Tris-base, 1.92M Glycine. Dilute to 1X with

buffer (10X)	distilled water and methanol (to a final concentration of 20% v/v)
TBS-Tween	25mM Tris-HCl pH7.6, 50mM NaCl, 0.1% v/v Tween 20
Stripping buffer	25mM glycine pH2 + 1% SDS in H <sub>2</sub> O

### 2.1.2. Primary Antibodies

**Table 2.3:** Primary Antibodies

Antibody	Company	Species	Dilution for WB	Dilution for IF
β-actin	Sigma-Aldrich	Mouse	1:5000	-
PAK4	Cell Signalling Technology	Rabbit	1:500	-
c-Met	Santa Cruz	Rabbit	1:500	-
c-Myc (9E10)	Santa Cruz	Mouse	1:1000	1:500
ERK1/2	Cell Signalling Technology	Rabbit	1:2000	-
FilaminA	Gentex	Rabbit	1:1000	-
GAPDH	Millipore	Mouse	1:10,000	-
GFP	Roche	Mouse	1:1000	-
Phospho-ERK (p44/42 MAPK, Thr202/Tyr204) (E10)	Cell Signalling Technology	Mouse	1:1000	-
EGFR	Cell Signalling Technology	Rabbit	1:500	-
PAK4	Cell Signalling Technology	Rabbit	1:500	-
PAK4/6	Cell Signalling Technology	Rabbit	1:1000	-
PAK6	Calbiochem	Rabbit	1:500	-
PAK6	Gnetex	Rabbit	1:2000	-
pFilaminA	Cell Signalling Technology	Rabbit	1:1000	-
RFP	BioVision	Rabbit	1:1000	-
Phospho-PAK4 (Ser474) PAK5 (Ser602) PAK6 (Ser560)	Cell Signalling Technology	Rabbit	1:500	-

### 2.1.3. Secondary Antibodies

**Table 2.4:** Primary Antibodies

Antibody	Company	Source	Dilution for WB	Dilution for WB
Horseradish peroxidase (HRP)-conjugated anti mouse	Dako	Goat	1:2000	-
Horseradish peroxidase (HRP)-conjugated anti rabbit	Dako	Goat	1:2000	-
AlexaFluor® 488 anti mouse	Invitrogen	Goat	-	1:200
AlexaFluor® 488 anti rabbit	Invitrogen	Goat	-	1:200
AlexaFluor® Rhodamine Phalloidin	Invitrogen		1:500	-

### 2.1.4. Plasmids

**Table 2.5:** Plasmid Constructs.

Construct	Source
c-Myc FilaminA	Dr. Mark Dodding, KCL, UK
c-Myc RhoV	Dr. Mark Dodding, KCL, UK
c-Myc RhoU	Dr. Pontus Aspenstrom
c-Myc RhoG	Dr. Pontus Aspenstrom
GFP-MyosinIIA	Dr. Michelle Peckham, Leeds, UK
GFP-FilaminA	Dr. David Calderwood, Yale, UK
GFP-RhoA (WT)	Prof. Anne Ridley, KCL, UK
GFP-RhoD	Prof. Anne Ridley, KCL, UK
GFP-PAK6 (WT)	Dr.Sally Fram, KCL, UK
RFP-PAK6	Dr.Sally Fram, KCL, UK
Myc-PAK6	Dr.Sally Fram, KCL, UK
Myosin IIA	Dr.Michelle Peckham, Leeds
GFP-PAK6 Kinase active (S531N)	Dr.Sally Fram, KCL, UK
GFP-PAK6 Kinase dead (K436A)	Dr.Sally Fram, KCL, UK
GFP Cdc42	Dr. Maddy Parsons, KCL, UK

## **2.2.Methods: Molecular Biology**

### **2.2.1. Transformation of *Escherichia coli* cells**

The heat shock method was used to transform *E.coli* (DH5 $\alpha$  strain) bacteria. The strain used varied between different assays. Competent *E.coli* cells stored at -80°C were thawed out on ice prior to transformation. 2  $\mu$ l of plasmid DNA was added to 50  $\mu$ L of the bacterial competent cells. These cells were then incubated on ice for 30 minutes followed by incubation at 42°C for exactly 20 seconds. The reaction mix was then placed on ice for 5 minutes. 950  $\mu$ L L-broth was then added to the reaction mix and then vials were incubated at 37°C in a shaking incubator for one hour at approximately 225rpm. Subsequently, 200  $\mu$ L of the transformation reaction was plated onto pre-warmed LB plates containing the appropriate antibiotic for plasmid selection (100  $\mu$ g/mL ampicillin or 30  $\mu$ g/mL kanamycin). The plates were inverted and incubated at 37°C overnight. The following day, colonies were picked and grown in L-broth containing the appropriate selection antibiotic.

### **2.2.2. Purification of plasmid DNA**

Plasmid DNA was isolated and purified from *E.coli* cells using either the Invitrogen Purelink<sup>TM</sup> HiPure plasmid DNA purification maxiprep kit, or the Purelink<sup>TM</sup> HiPure Plasmid Filter Mini- prep kit, dependent on the DNA yield required. L-broth supplement with the appropriate antibiotic added was inoculated with transformed bacteria and incubated at 37°C overnight with shaking. The concentrations of antibiotic used were 30  $\mu$ g/mL for kanamycin and 100  $\mu$ g/mL for ampicillin. The plasmid DNA eluted in TE buffer and stored at -20°C. DNA was purified as per the manufacturer's protocol and resulting DNA resuspended in TE buffer was stored at -20°C for future use.

### **2.2.3. Determination of DNA concentration**

DNA concentration was determined using a Nano-Drop system. A 1 $\mu$ l sample was pipetted onto the end of a fibre optic cable and the optical density (OD) was measured at 260 nm. The DNA concentration in ng/ $\mu$ L was automatically determined



## **2.3.Methods: cell biology**

### **2.3.1. Cell culture**

The breast cancer cell lines MDA-MB-231 (kind gift from Prof. Anne Ridley), MCF-7 (ATCC), HT29 human colon cancer and HEK 293 (ATCC) cells were grown in Dulbecco's modified eagles medium DMEM containing 10% foetal bovine serum (FBS) and 100U/mL penicillin and 100 µg /mL streptomycin (Gibco). MCF10A (ATCC) were grown in DMEM/F12 containing 5% foetal bovine serum (FBS) and 100U/mL penicillin and 100 µg/mL streptomycin with 100µL EGF, 250µL Hydrocortizone, 50µL CholeraToxin and 500µL Insulin. DU145 and PC3 prostate cancer cells (obtained from Claire Wells, King's College London) were cultured in in RPMI-1640 media supplemented with 10% foetal bovine serum (FBS) and 100U/mL penicillin/streptomycin. A375 melanoma cell lines were in cultured Dulbecco's modified eagle's medium: nutrient F-12 ham (DMEM F-12) containing 2.5mM L-glutamine.

All media were warmed before use to 37°C. Cells were maintained in humidified incubator at 37°C with 5% CO<sub>2</sub> and passaged at 80% confluent levels. During cell passaging the growth medium was removed and the cells were washed with sterile phosphate buffered saline (PBS) and trypsinised with 0.5% Trypsin/Ethylenediaminetetraacetic acid (EDTA). The cell pellet resuspended in growth media and suspended cells were placed in new tissue culture flask at appropriate dilution.

### **2.3.2. Thawing and freezing cells**

To thaw cells, a cryovial was taken from liquid nitrogen and thawed rapidly at 37°C in a water bath. The cells were then transferred to 5 mL (T25 flask) of cell specific medium in a tissue culture flask. The medium was changed the next day and cells were cultured using the method illustrated in 2.3.1

To freeze cell lines, cells were detached using trypsin and transferred into cryovials in a 90% FBS, 10% DMSO solution. Cryovials were placed in a cryo-freezing container and stored at -80°C to allow slow freezing overnight. The cells were then transferred to liquid nitrogen for long-term storage.

### 2.3.3. Lipofectamine transfection

The Lipofectamine was used to transfect MDA-MB-231 and MCF-7 Cells. The cells were seeded at density  $1 \times 10^5$  /mL then incubated at 37° for 24 hours. On the day of transfection, the medium was aspirated from the cells and replaced with serum free OptiMEM medium, free from antibiotics. The transfection mixture was then made up in OptiMEM serum free media, details of which are outline in Table 2.6.

**Table 2.6:** Lipofectamine transfection reaction mix.

	<b>2cm tissue culture plate (2 mL)</b>	<b>10cm tissue culture plate (10 mL)</b>
Tube A	3µl of lipofectamine  100µl serum-free OptiMEM media	15µl of lipofectamine  100µl serum-free OptiMEM media
Tube B	3 µg of DNA  100 µl serum-free OptiMEM media	15 µg DNA  500 µl OptiMEM media

The mixtures were left at room temperature for 5 minutes. Next this, the contents of tube A added to the tube B mix and then incubated at room temperature for 15 minutes. The cells were then incubated with the transfection reagents and maintained at 37°C for 6 hours. Then, optimum media replaced with growth media.

### 2.3.4. Calcium Phosphate Transfection

The calcium phosphate transfection kit was used to transfect HEK293 cells. Cells were seeded at a density of  $1 \times 10^5$ /mL and incubated at 37°C overnight. On the day of transfection, 3-4 hours prior to the addition of the reaction mix, the media was aspirated and replaced with fresh medium. The reaction mix was set up using the conditions described in Table 2.7.

**Table 2.7:** Calcium phosphate transfection reaction mix.

	2cm tissue culture plate (2 mL)	10cm tissue culture plate (10 mL)
<b>Tube A</b>	7.2µL 2M CaCl <sub>2</sub> 4µg DNA Make up to 60µL with sterile water	30µL 2M CaCl <sub>2</sub> 20µg DNA Make up to 300µL with sterile water
<b>Tube B</b>	60µL 2x Hepes buffered saline	300µL 2x Hepes buffered saline

The contents of tube A added to the tube B, with aeration. This transfection mixture was then incubated at room temperature for 30 minutes, before being added dropwise to the cells.

### 2.3.5. HiPerfect siRNA transfection

Cells were plated at density  $1 \times 10^4$  /mL the day before transfection in full growth media. The following day, 60nM short interfering RNA (siRNA) oligonucleotides and 12µL HiPerfect transfection reagent were added to 97µL optiMEM and incubated at room temperature for 20 mins before being added dropwise to the cells. Cells were then incubated at 37°C for different time frames depending on the assay. Equal concentrations of a control siRNA were used in each experiment. The siRNA oligonucleotides used in this study (including the target sequence) are shown in Table 2.8.

**Table 2.8:** The Sequences of siRNAs used in this study.

Oligo Name	Target sequence
Control siRNA	AAT TCT CCG AAC GTG TCA CGT
PAK6 Oligo 1	GGCUAUUCCGAAGCAUGUTT
PAK6 Oligo 2	GCACCAAUAGGCAUGGAAU

### 2.3.6. Inhibitors

Panobinostat LBH-589 (molecular formula:  $C_{21}H_{23}N_3O_2$ ) is known to be a highly selective inhibitor of histone deacetylase (HDAC), inducing hyperacetylation of core histone proteins, which may result in modulation of cell cycle protein expression, cell cycle arrest in the G2/M phase and apoptosis and has been shown to inhibit tumour cell proliferation *in vitro*. Blebbistatin (molecular formula:  $C_{18}H_{16}N_2O_2$ ) is identified as high affinity and selectivity small molecule inhibitor myosin II.

RhoA (C3) is known to inhibit RhoA by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase. ROCK H-1152 (molecular formula:  $C_{16}H_{21}N_3O_2S \cdot 2HCl$ ) is known to be more specific, potent and membrane-permeable inhibitor of Rho-kinase. A stock solution, at a concentration of 10  $\mu$ M, was made by diluting in DMSO for panobinostat and blebbistatin and in distilled water for ROCK and RhoA inhibitors. Aliquots were stored at -20 °C until required. For use in all assays the stock solution was diluted further in culture medium to establish a working concentration of 5  $\mu$ M. An equal volume of DMSO and water was used as a control in all experimental procedures.

### 2.3.7. MTT Proliferation Assay

Cells were seeded onto plastic or matrix-coated 96 well plates at a cell density of  $3 \times 10^3$ /mL in DMEM media. Media was aspirated from each well and washed once with sterile PBS and replaced with 50  $\mu$ L (5mg/mL) methyl thiazolyldiphenyl tetrazolium bromide (MTT) diluted in serum free DMEM media. Cells were incubated in the presence of the MTT at 37°C, in 5% CO<sub>2</sub> for 3 hours. Following this incubation, the MTT solution was carefully removed and 50  $\mu$ L DMSO was added. The cells were mixed thoroughly and the absorbance at 540 nm was measured using an Alpha-Fusion plate reader.

### 2.3.8. 3D Spheroid Invasion Assay

To form spheroids, MDA-MB-231 cells were seeded into a 96-well U-bottomed suspension culture plate at density of  $2.5 \times 10^2$ - $10 \times 10^2$  in 100 of DMEM media, containing 5% FBS and 0.32% methylcellulose. Cells were incubated at 37°C in 5%

CO<sub>2</sub>, for three days to enable a spheroid mass to form. On the third day, collagen I matrix (from bovine) was prepared on ice and 400µL was added to a 24 well plate and allowed to polymerise for 2 hours at 37°C in 5% CO<sub>2</sub>. Following this, another 400µL of collagen I matrix was added on to the polymerized collagen I layer and the spheroid was transferred directly into this layer. Images were taken at day 0 and after 17-24 hours using an Olympus Ix71 microscope with Image-Pro Plus 7.0 software (x10 objective).

### **2.3.9. Preparation of coverslips for Immunofluorescence**

13 mm round glass coverslips (VWR, UK) were placed in a solution containing 40% of 1 M hydrochloric acid (HCL) and 60% of 70% ethanol at room temperature overnight in the fume hood. Following this, the coverslips were rinsed three times in distilled water and boiled. Then, rinsed a further six times with distilled water and then autoclaved.

#### **2.3.9.1. Rat tail collagen type I coated coverslips**

Type I rat tail collagen was diluted to 50µg/mL in 0.02M glacial acetic acid, which had been filter sterilized. The dilute collagen solution was added and incubated at room temperature for one hour. The collagen was then aspirated and washed twice with sterile PBS before cells were seeded.

#### **2.3.9.2. Fibronectin coated coverslips**

The fibronectin was diluted to a final concentration of 10µg/mL in sterile PBS was then added and incubated at room temperature for 45 minutes. The fibronectin was then aspirated and washed twice with sterile PBS before cells were seeded.

### **2.3.10. Immunofluorescent labelling**

Cells were seeded onto sterilise coverslips at appropriate density. The following day, cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Coverslips were washed three times with phosphate buffer saline (PBS) plus calcium and maganesium (Oxoid,UK) before being permeabilised with 0.2% Triton X-100 for 5 minutes. Cells were then washed three times with PBS. For F-actin staining cells were incubated at room temperature for 1 hour with TRITC-Phalloidin or Alexa Fluor 488 conjugated phalloidin. For nuclei staining were stained by adding DAPI. Coverslips were then washed twice with PBS

and once with dH<sub>2</sub>O. Then, cells fixed to microscope slide and mounted in 10  $\mu$ l FluorSave<sup>TM</sup> Reagent.

For Myc-PAK6 and Myc-Filamin, cells were blocked in 3% BSA-PBS for 30 minutes and incubated in primary antibody, diluted in 3% BSA for 2 hours at room temperature in the dark. Then, washed three times in PBS and incubated with the appropriate secondary antibody and DAPI diluted in 3% BSA-PBS for one hour at room temperature in the dark. Coverslips were then mounted onto microscope slides using FluorSave, left at room temperature in the dark overnight and stored at -20°C until being imaged. The fluorescence was visualized using on Olympus IX71 microscope with Image-Pro Plus 7.0 software.

### **2.3.11. Growth factor stimulation**

#### **2.3.11.1. Stimulation of cells on 6-wells plates**

MDA-MB-231 and MCF-7 cells were seeded onto a 6-well plate at a density of  $1 \times 10^5$  in 2 mL of growth media and incubated at 37°C overnight. Cells were then incubated for 24 hours in starve media; Dulbecco's modified eagles medium DMEM containing 0.5% FBS and 100U/mL penicillin and 100  $\mu$ g/mL streptomycin. Then, starved cells stimulate with (10ng/mL) HGF or (100ng/mL) EGF at designed time points. Cells were lysed in lysis buffer NP40 supplemented with protease inhibitors on ice for 10 minutes and centrifuged at 13000 rpm for 10 minutes to remove cell debris. Lysates were immunoblotted for the desired protein to quantify the changes in phosphorylation levels of PAK6 following HGF or EGF stimulation. The intensity of each phospho-protein and loading band was determined using Image J.

#### **2.3.11.2. Stimulation of cells on coverslips**

MDA-MB-231 and MCF-7 cells were seeded onto coverslips at density of  $2 \times 10^4$  /mL in 1mL of growth media and incubated at 37°C overnight. Cells were then incubated for 24 hours in starve media; Dulbecco's modified eagles medium DMEM containing 0.5% FBS and 100U/mL penicillin and 100  $\mu$ g /mL streptomycin. Then, starved cells stimulate with (10 ng/mL) HGF or (100 ng/mL) EGF For 15 minutes. Moreover, MCF-7 stimulated for 24 hour. Then, cells washed twice with PBS before being fixed with 4% PFA and immunostained as described in section 2.3.10.

### **2.3.12. Image processing and Cell shape analysis**

Coverslips were viewed on Olympus IX71 microscope with Image-Pro Plus 7.0 software. Image J software was used to determine the morphology of each cell by drawing around individual cell. The data were then processed to measure the area, elongation ratio. The cell elongation was calculated as a relative value of the longest axis to the shortest axis of a cell, subtracted from 1 reference. The average value (mean) and standard error were calculated. This process was repeated for 90 cells over three experiments.

## **2.4. Methods: Cell Biochemistry**

### **2.4.1. Preparation of cell lysates**

When cells were approximately 70% confluent, they were washed twice in PBS and lysed for 10 minutes on ice in 100  $\mu$ l 0.5% NP-40 lysis buffer supplemented with 1mM DTT, 1 $\mu$ g/mL aprotinin, 10 $\mu$ g/mL leupeptin, 1mM PMSF, 50mM sodium fluoride and 1mM sodium orthovanadate for six well plates and 1000  $\mu$ l for 10 CM plates. Cells were scraped and transferred to eppendorf tubes and centrifuge at 13,000g for 10 minutes at 4°C to remove cell debris. The supernatant was transferred to a fresh eppendorf and samples prepared through the addition of a 6x Laemmli buffer (diluted to 1x) before samples were boiled for 3 minutes. Whole cell lysate samples were then subsequently stored at -20 °C until use.

### **2.4.2. Gel electrophoresis and immunoblotting**

Cellular proteins were separated on 6.5-12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels at 125 V in running buffer for 1-2 hour. Proteins were electroblotted onto nitrocellulose membrane at 70-100 volts for 1-2 hours (dependent on the percentage gels used) in transfer buffer. The membrane was then blocked in 5% skimmed milk powder in 0.1% TBS-Tween20 or 5% BSA in 0.1% TBS-Tween20, for one hour at room temperature to block non specific binding. The membrane was incubated in primary antibody overnight at 4 °C. The membrane was washed three times for 10 minutes each in TBST to remove unbound primary antibody. Following this, membrane was incubated with secondary antibody conjugated with horseradish peroxidase at double the dilution of primary antibody in TBST with milk for one hour at room temperature. Proteins were detected using Pierce enhance chemiluminescence (ECL) kit.

### **2.4.3. Stripping of Nitrocellulose Membranes**

To allow reprobing, the nitrocellulose membranes were incubated in stripping buffer twice for 15 minutes each at room temperature. Membranes were then washed twice for 10 minutes in TBS-T Tween20. Consequently, the blots were blocked for 1 hour in 5% milk and incubated overnight with primary antibodies at 4 °C. Following day, the blot was incubated for secondary antibody as previously stated.

### **2.4.4. Immunoprecipitation**

Lysates of HEK 293 and MDA-MB-231 cells were prepared using IP lysis buffer with inhibitors. 50 µl each lysate was kept for immunoblotting as a loading control. 3 µl of appropriate IP antibody was added to the lysis supernatant and incubated on a rotating wheel overnight at 4 °C. Following day, protein A or G beads (depend on the antibody) were washed three times in appropriate volume lysis buffer with inhibitors, before to adding 30 µl of beads to each IP sample. The samples were then incubated for 1 hour on a rotating wheel at 4 °C. Each samples following incubation, washed three times with lysis buffer with inhibitors. Gel sample buffer was added to each sample and boiled at 90 °C for 3 minutes.

### **2.4.5. GFP-TRAP**

Lysates of HEK 293 and MDA-MB-231 cells were prepared using GFP-TRAP lysis buffer with inhibitors. Prior to the adding the GFP-TRAP, 10 µl of beads were washed three times and centrifuge at 2500xg for two minutes. Following that, the supernatant added to the beads and incubated for 2 h rotating at 4°C. 50 µl of each lysate was kept for immunoblotting as a loading control. After the incubation, the beads were washed three times with 1 mL of GFP TRAP wash buffer, at 4°C. The supernatant was aspirated very carefully using a pipette. After the last wash, the Sample Buffer were added and samples were boiled at 90°C for 3 minutes.



#### **2.4.6. Densitometry**

For western blotting, densitometric analysis of specific bands was performed using ImageJ software. In order to obtain a semi-quantitative measurement of the level of total protein, all band densities were normalised to a loading control. In most circumstances,  $\beta$ -actin was used as a loading control and was detected on the same membrane as the protein of interest.

#### **2.4.7. Statistics**

Statistical analysis was carried out where indicated using data of three independent experiments. Statistical significance was calculated using an unpaired Student's t-test, or where specified using one-way ANOVA (analysis of variance). Values were considered statistically significant if the P-value was  $<0.05$ . Error bars represent standard error of the mean (SEM).

## **Chapter 3:**

### **Investigation the role of PAK6 in Breast Cancer cell lines**

### **3. Chapter 3: Investigation the role of PAK6 in Breast cancer**

#### **3.1. Introduction**

Our group published that HGF stimulation increases PAK6 autophosphorylation and an increase in PAK6 expression also leads to cell-cell dissociation in response to HGF. Moreover, overexpression of PAK6 induces morphological changes when compared to control cells (Fram et al., 2014). Tumour metastasis involves multiple steps, and cell migration is one of the key steps in the development of this complex process (Spano et al., 2012). Growth factors can promote cell migration; both *in vivo* and *in vitro*. HGF or EGF are known to activate a variety of intracellular signalling cascades via stimulation of c-Met and EGFR receptors, respectively. Many of these downstream signalling cascades participate in the regulation of pathways related to actin cytoskeletal reorganization (Ridley et al., 1995), cell motility and/or invasive growth of tumour cells, which provide the hallmarks of metastatic cancer (Maulik et al., 2002). HGF or EGF signalling pathways have been shown to be involved in the spread of prostate cancer or breast cancer, respectively (Kim and Muller, 1999). HGF induces invasiveness of prostate cancer cells *in vitro* (Parr et al., 2010), and increased expression of c-Met/HGF in breast carcinoma have been correlated with poor prognosis and increased cell proliferation (Taniguchi et al., 1994). HGF also increases the invasiveness of breast cancer cells (Woodbury et al., 2002) and the serum levels of HGF are elevated in breast cancer patients. The elimination of malignant breast tumours also results in a reduction in serum HGF levels (Woodbury et al., 2002, Yamashita et al., 1994). Furthermore, HGF can drive cell migration and invasion in breast cancer cell lines (Mine, 2003).

PAK family proteins can be activated downstream of HGF or EGF, and are known effectors of Rho Family GTPases (Wells, 2002, Yang et al., 2011). Indeed, both PAK1 and PAK4 are required for the HGF-induced migration of prostate cancer cells (Ahmed et al., 2008, Bright et al., 2009), and activated PAK4 induces cell rounding following the HGF stimulation of MDCK cells (Wells, 2002). EGFR also plays a role in the activation of PAK1 and PAK4 (Beier et al., 2008, Siu et al., 2010) and EGF stimulates MDA-MB-231 cell migration *in vitro* via activation of PAK1 (Yang et al., 2011).

PAK6 was first detected in a screen to identify proteins that interact with the androgen receptor (AR) (Yang et al., 2001). It is known that endogenous PAK6 is expressed in DU145 prostate cancer cells (Fram et al., 2014, Wells and Jones, 2010, Yang et al., 2001). The overexpression of wild type PAK6 cells were significantly elongated and highly expressing cells became rounded in prostate cells. In addition, the overexpression of activated PAK6 also induced cell elongation, although not to the same degree as that observed in cells overexpressing wild type PAK6. Again highly expressing cells become rounded (Fram et al., 2014). This is unusual as the majority of PAK induced cell morphological changes have been detected only when the constitutively active forms of PAK1, PAK2 or PAK4 are utilized (Wells, 2002, Manser et al., 1997, Zeng et al., 2000, Qu et al., 2001).

PAK6 interacts with the active form of Cdc42, but this does not enhance the kinase activity of PAK6 (Schantz et al., 2004). PAK6 kinase activity can be regulated by the MAPK signalling pathway (Kaur et al., 2005). Phosphorylation by p38 MAPK and mitogen activated protein kinase kinase 6 (MKK6) can stimulate PAK6 (Kaur et al., 2005). Furthermore, the autophosphorylation of PAK6 at serine 560 in the activation loop is required for MKK6-facilitated activation. The AR may also play a part in regulating PAK6 kinase activity in prostate cancer cells (Lee et al., 2002). However, there is much less known about the regulation and activation of PAK6 in breast cancer.

In this chapter, the expression level of PAK6 in breast cancer cell lines was analysed, and the possibility that PAK6 might be activated downstream of HGF or EGF was tested using MDA-MB-231 and MCF-7 breast cancer cells. The morphological impact of PAK6 overexpression was also investigated in the breast cancer cell lines.

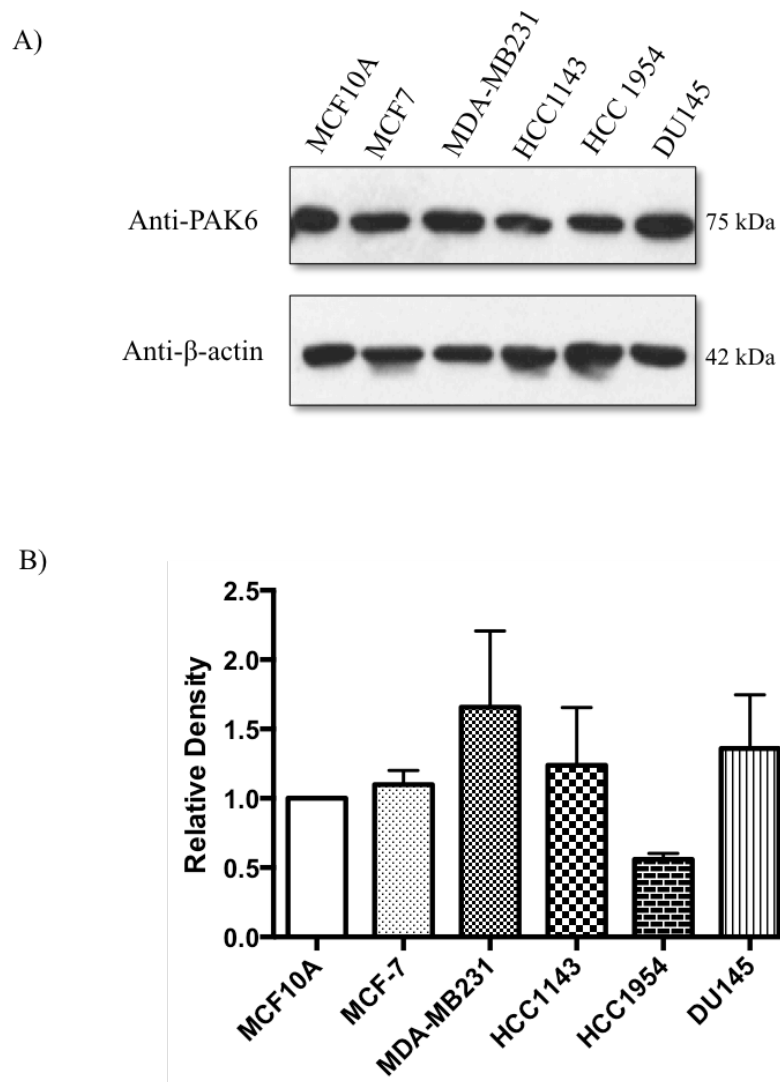
### 3.2. Results

#### 3.2.1. PAK6 is endogenously expressed in MDA-MB-231 and MCF-7 cells

PAK6 expression has been linked to prostate cancer invasiveness (Wen et al., 2009). In this study, they also demonstrated that the depletion of PAK6 expression inhibited the growth of the prostate cancer cells. Consequently, PAK6 expression was tested in a panel of breast cell lines, as illustrated in the (Table 3.2); a non-tumorigenic epithelial cell line (MCF10A), non-invasive breast cancer cells (MCF-7), invasive human breast cancer cells (MDA-MB-231) and (HCC1954), poorly differentiated breast cancer cells (HCC1143) and DU145 prostate cancer cells were used as a positive control. In these cell lines, PAK6 was found to be present at 75 kDa using specific PAK6 antibody from Gentex (Figure 3.3). This antibody detected a single protein band at the correct size for PAK6. The Gentex antibody did not appear to cross-react with PAK4 or PAK5, which run with a different apparent molecular weight. This antibody was therefore selected for future studies, and PAK6 expression was detected in all of the cell lines, both colony and single cells (Figure 3.3). Although not statistically significant, there appeared to be potential and slightly higher expression in MDA-MB-231 cells.

**Table 3.1:** Panel of breast and prostate cell lines.

	Cell Type	Tissue	Disease
HCC1954	Epithelial	mammary gland; breast/duct	TNM stage IIA, grade 3, ductal carcinoma
DU145	Epithelial	prostate; derived from metastatic site: brain	Carcinoma
MDA-MB-231	Epithelial	mammary gland/breast; derived from metastatic site: pleural effusion	Adenocarcinoma
MCF-7	Epithelial	mammary gland, breast; derived from metastatic site: pleural effusion	Adenocarcinoma
MCF 10A	Epithelial	mammary gland/breast	fibrocystic disease
HCC1143	Epithelial	breast; mammary gland/duct	TNM stage IIA, grade 3, primary ductal carcinoma

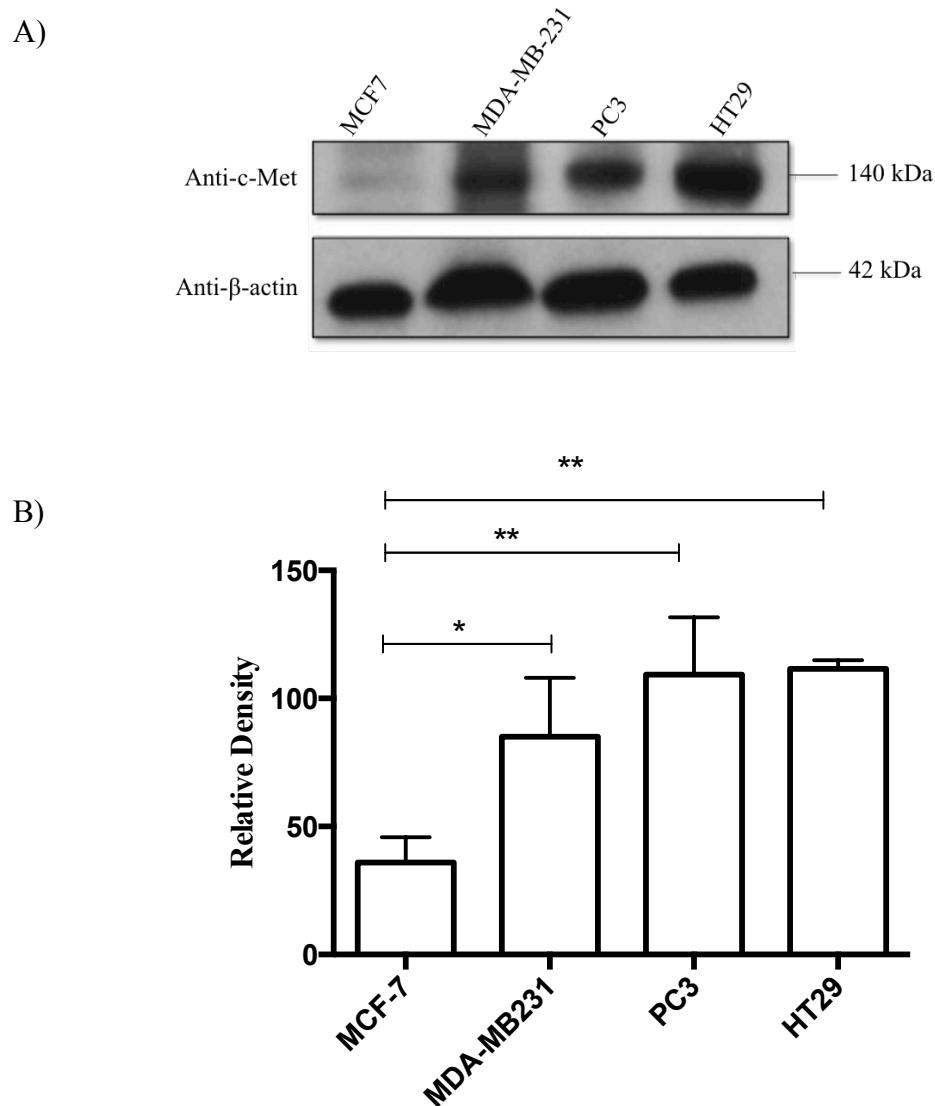


**Figure 3.1: PAK6 expression in breast and prostate cell lines.** A) Western blotting was used to analyze the expression of PAK6 in a panel of cell lines. Cell lysate were immunoblotted for PAK6 expression using Gentex PAK6 antibody or probed for  $\beta$ -actin as a loading control. B) The blot shown is representative of three independent experiments, which were quantified through densitometry. Bars represent the mean value of  $\pm$  S.E.M.

### **3.2.2. Expression of growth factor receptors in panel of cancer cell lines**

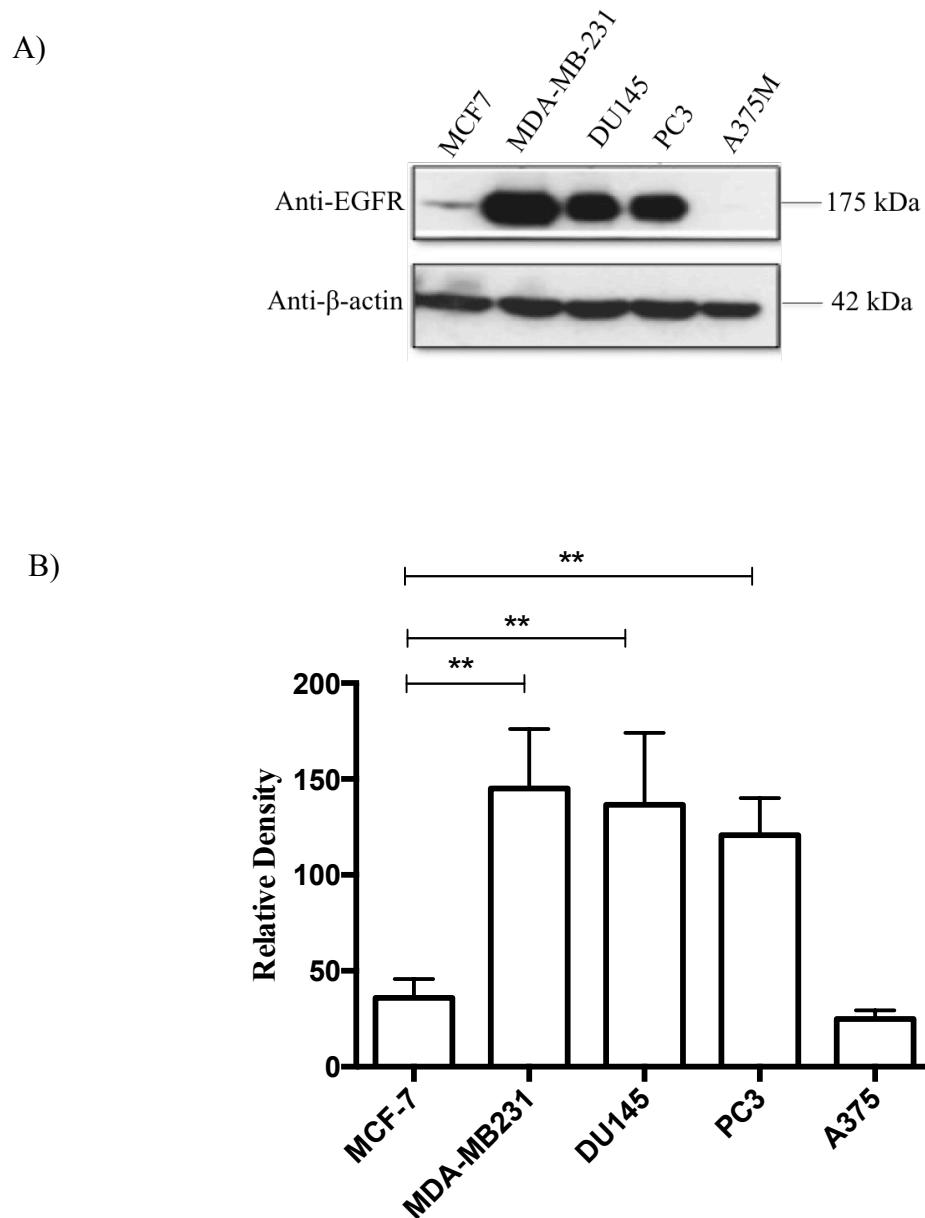
HGF and EGF signalling pathways have both been implicated in breast cancer progression (Harrison et al., 2013, Natan et al., 2014). Furthermore, previous work has suggested that PAK family kinases can be activated downstream of growth factor stimulation (Bright et al., 2009, Wells, 2002). Initial experiment analysed the endogenous expression of the receptors for Hepatocyte Growth Factor (HGF)/c-Met and Epidermal Growth Factor (EGF)/EGFR in selected cell lines. Cell lines were selected that were known to be positive for PAK6 expression as a positive control. Detectable c-Met protein expression was observed in PC3, HT29 and MDA-MB-231 malignant cell lysates cells. In contrast, MCF-7 cells appeared to express c-Met at much lower levels (Figure 3.4).

Additional immunoblotting experiments showed that EGFR was expressed in all of the cell lines analysed and the highest levels of expression were detected in MDA-MB-231 cells. In contrast, A375 and MCF-7 cells appeared to have lower expression of EGFR (Figure 3.5).



**Figure 3.2: c-Met expression in panel of cancer cell lines.** A) Western blotting was used to analyze the expression of c-Met in a panel of cell lines as indicated. Cell lysates were immunoblotted for c-Met expression or  $\beta$ -actin as loading control. B) The blot shown is representative of three independent experiments, which were quantified through densitometry. The molecular weight of c-Met is 140 kDa and  $\beta$ -actin 42 kDa. Statistical significance was calculated using *t*-test, \* $P < 0.05$ , \*\* $P < 0.005$ , n.s.=not significant. Bars represent the mean value of  $\pm$  S.E.M.





**Figure 3.3: EGFR expression in panel of cancer cell lines.** A) Western blotting was used to analyze the expression of EGFR in a panel of cell lines as indicated. Cell lysates were immunoblotted for EGFR expression or β-actin as loading control. B) The blot shown is represent of three independent experiments, which were quantified through densitometry. The molecular weight of EGFR is 175kDa and β-actin 42 kDa. Statistical significance was calculated using *t*-test, \*\* $P < 0.005$ , n.s=not significant. Bars represent the mean value of  $\pm$  S.E.M.

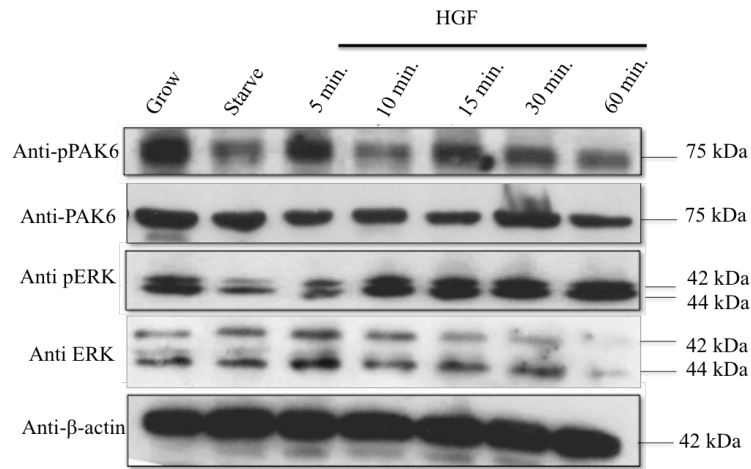
### **3.2.3. Growth factor stimulation did not modify PAK6 autophosphorylation**

Having established that the MDA-MB-231 and MCF-7 cell lines selected for this project express PAK6, c-Met and EGFR, these two cell lines were selected to test the ability of HGF and EGF to activate PAK6. The effect of HGF or EGF stimulation on PAK6 autophosphorylation levels was therefore investigated using a phospho-specific antibody from Cell Signalling Technology. This antibody detects phosphorylated PAK4 on serine 474, phosphorylated PAK5 on serine 602 and phosphorylated PAK6 on serine 560 within the kinase domain. This residue in PAK6 is thought to be an autophosphorylation site homologous to threonine 423 (T423) in PAK1 (Schantz et al., 2004) and serine 474 (S474) in PAK4 (Abo et al., 1998).

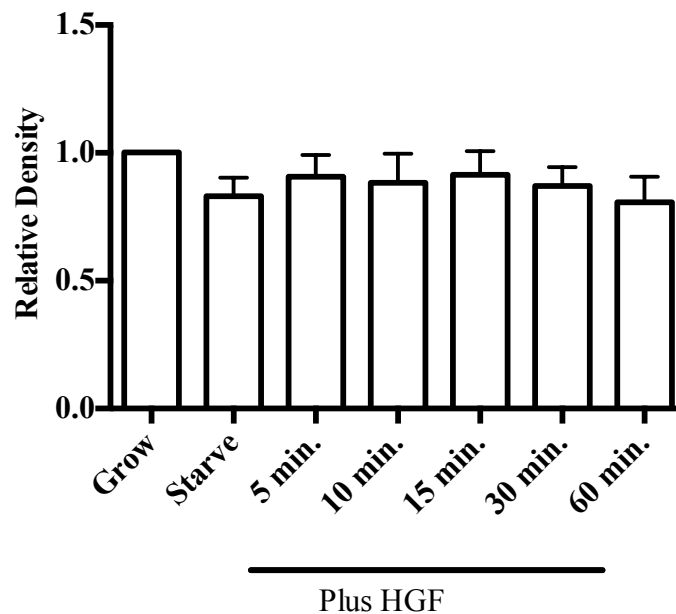
In MDA-MB-231 cells, the level of PAK6 phosphorylation decreased when the cells were serum starved and appeared to increase slightly following 5 minutes of HGF stimulation, when compared to the starved cells (Figure 3.6 A). However, this change in PAK6 phosphorylation levels was not significant (Figure 3.6 B). The blot was also probed for phospho-ERK to confirm efficient c-Met signalling downstream of HGF. Analysis beyond 5 minutes suggested that there was no significant increase in the PAK6 phosphorylation levels upon HGF stimulation (Figure 3.6). With regards to EGF stimulation, results show that there is no significant difference in the PAK6 phosphorylation level following EGF stimulation (Figure 3.7) in MDA-MB-231 cells despite a robust phosphoERK response.

Consistent with the results from the MDA-MB-231 cells, no change in PAK6 phosphorylation levels were detected in MCF-7 cells following stimulation with either HGF or EGF (Figure 3.8 and Figure 3.9), although increases in the level of phosphoERK were detected.

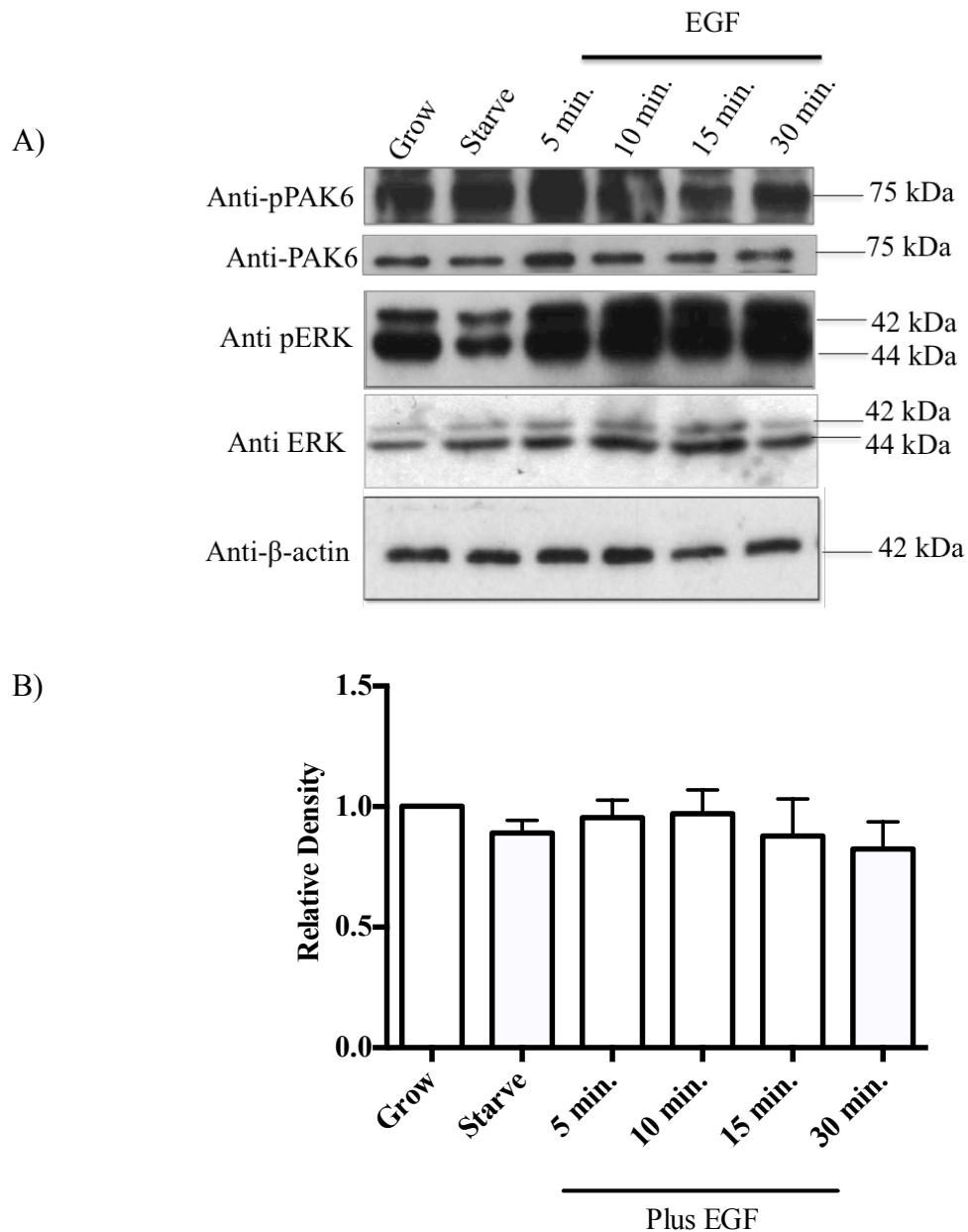
A)



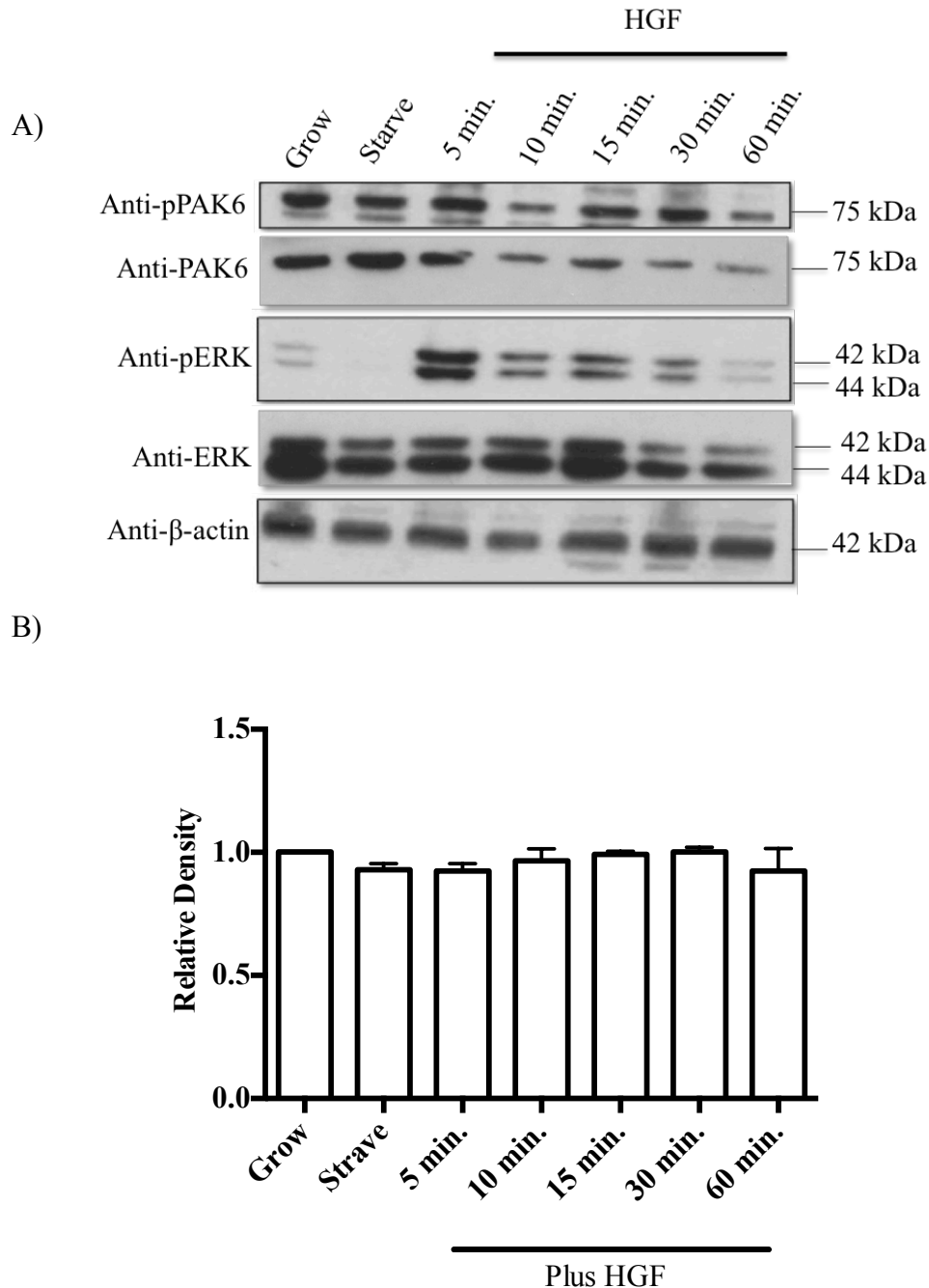
B)



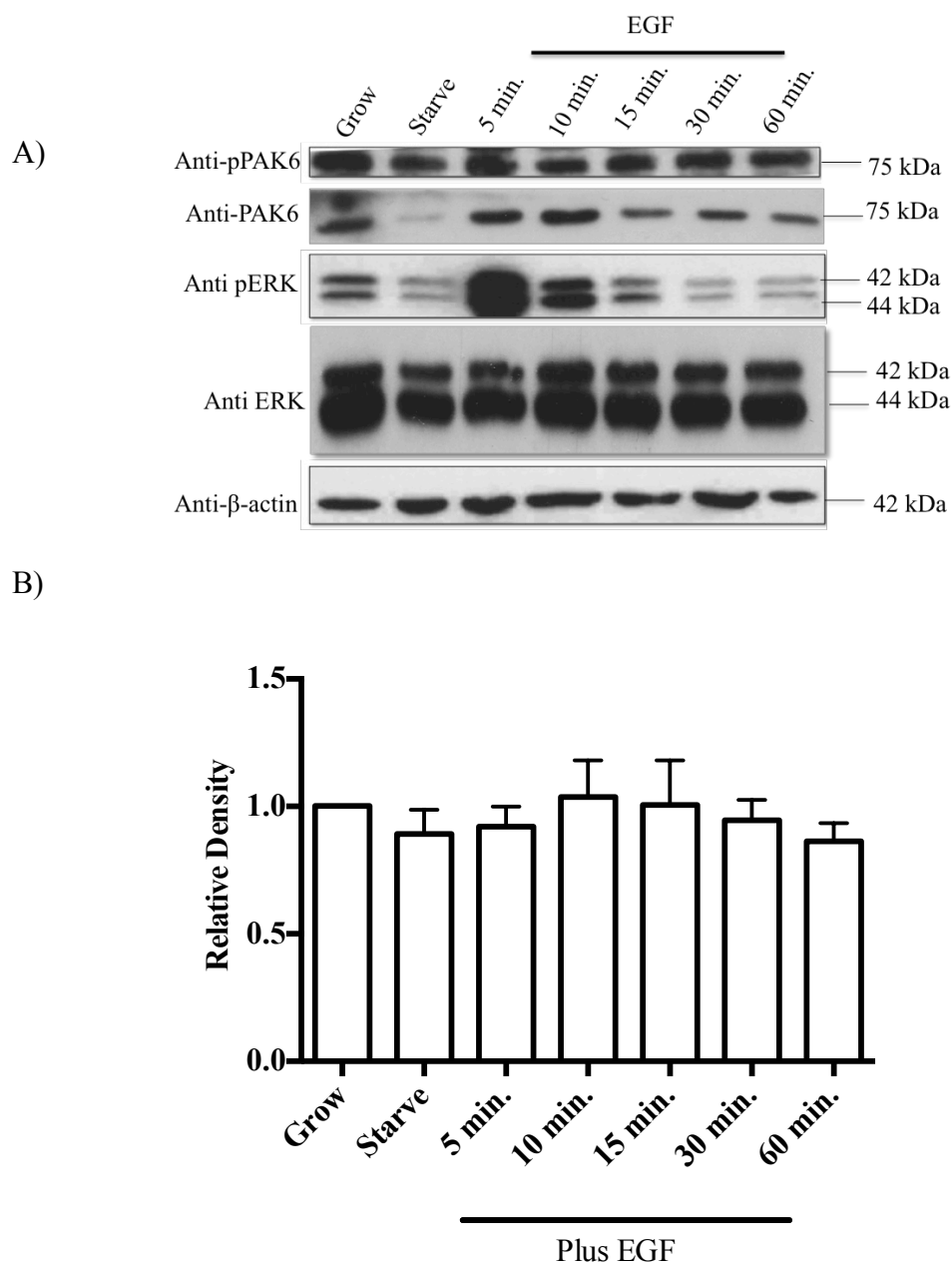
**Figure 3.4: HGF stimulation has no effect on PAK6 autophosphorylation levels in MDA-MB-231 cell line.** A) MDA-MB-231 cells were seeded and were serum-starved overnight after plating for 24 hours. Cells were then left unstimulated (starve) or stimulated with HGF (10 ng/mL) for 5, 15, 30 and 60 minutes prior to lysis. Lysates were immunoblotted for levels of PAK6 autophosphorylation at serine 560 using a phospho-PAK4/PAK5/PAK6 antibody or probed for phospho ERK using an anti-pERK antibody. Blots were re-probed for  $\beta$ -actin. The molecular weight for pPAK6 is 75 kDa, pERK 24/44 kDa and  $\beta$ -actin 42 kDa. B) Changes in PAK6 autophosphorylation levels at serine 560 were quantified. Bars represent the standard error of the mean, which calculated over three independent experiments.



**Figure 3.5: EGF stimulation has no effect on PAK6 autophosphorylation levels in MDA-MB-231 cell lines.** A) MDA-MB-231 cells were seeded and were serum-starved overnight after plating for 24 hours. Cells were then left unstimulated (starve) or stimulated with EGF (100 ng/mL) for 5, 15, 30 and 60 minutes prior to lysis. Lysates were immunoblotted for levels of PAK6 autophosphorylation at serine 560 using a phospho-PAK4/PAK5/PAK6 antibody and probed for phospho ERK using an anti-pERK antibody. Blots were re-probed for β-actin. The molecular weight for pPAK6 is 75 kDa, pERK 24/44 kDa and β-actin 42 kDa. B) Changes in PAK6 autophosphorylation levels at serine 560 were quantified. Bars represent the standard error of the mean, which calculated over three independent experiments.



**Figure 3.6: HGF stimulation has no effect on PAK6 autophosphorylation levels in MCF-7 cell line.** A) MCF-7 cells were seeded and were serum-starved overnight after plating for 24 hours. Cells were then left unstimulated (starve) or stimulated with HGF (10 ng/mL) for 5, 15, 30 and 60 minutes prior to lysis. Lysates were immunoblotted for levels of PAK6 autophosphorylation at serine 560 using a phospho-PAK4/PAK5/PAK6 antibody and probed for phospho ERK using an anti-pERK antibody. Blots were re-probed for β-actin. The molecular weight for pPAK6 is 75 kDa, pERK 24/44 kDa and β-actin 42 kDa. B) Changes in PAK6 autophosphorylation levels at serine 560 were quantified. Bars represent the standard error of the mean, which calculated over three independent experiments.



**Figure 3.7: EGF stimulation has no effect on PAK6 autophosphorylation levels in MCF-7.** A) MCF-7 cells were seeded and were serum-starved overnight after plating for 24 hours. Cells were then left unstimulated (starve) or stimulated with EGF (100 ng/mL) for 5, 15, 30 and 60 minutes prior to lysis. Lysates were immunoblotted for levels of PAK6 autophosphorylation at serine 560 using a phospho-PAK4/PAK5/PAK6 antibody and probed for phospho ERK using an anti-pERK antibody. Blots were re-probed for  $\beta$ -actin. The molecular weight for pPAK6 is 75 kDa, pERK 24/44 kDa and  $\beta$ -actin 42 kDa. B) Changes in PAK6 autophosphorylation levels at serine 560 were quantified. Bars represent the standard error of the mean, which calculated over three independent experiments.

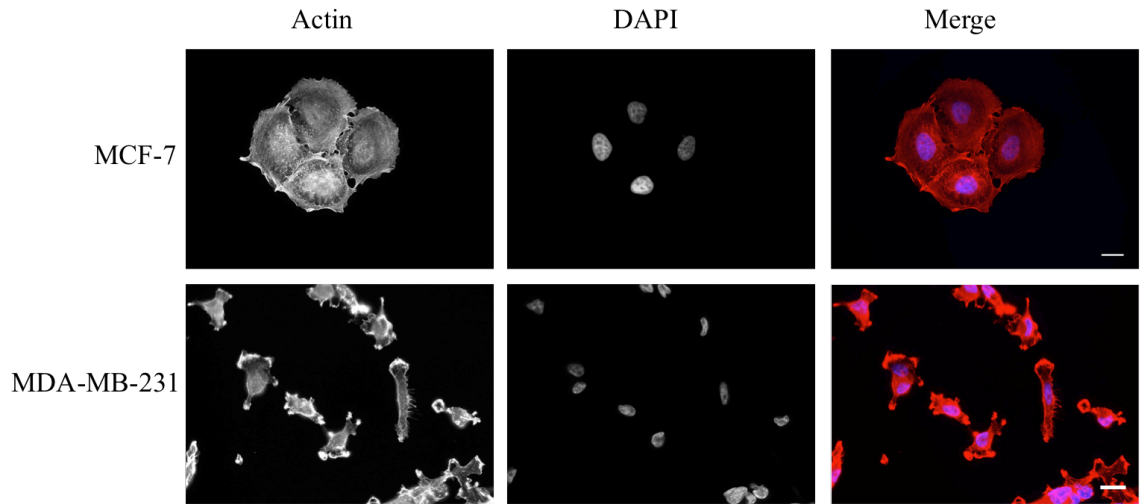
### **3.2.4. Cell morphology of breast cancer cell lines**

To complement the protein expression studies, the impact of PAK6 overexpression on cell morphology was investigated. Prior to initiating PAK6 overexpression experiments, the cell morphology of MDA-MB-231 and MCF-7 cells was characterised under basal and growth factor stimulated conditions. Cells were stained for F-actin using phalloidin and morphology analysed using Image J software. Illustrative staining of each cell line under basal conditions is represented in Figure 3.10 A. The cell area and cell elongation were calculated for both cell lines, and they were found to differ in both cell area and cell shape (Figure 3.10 B). Elongation values were found to be higher in MDA-MB-231 cells compared to MCF-7 cells. However, MCF-7 cells exhibited a larger area than MDA-MB-231 (Figure 3.10).

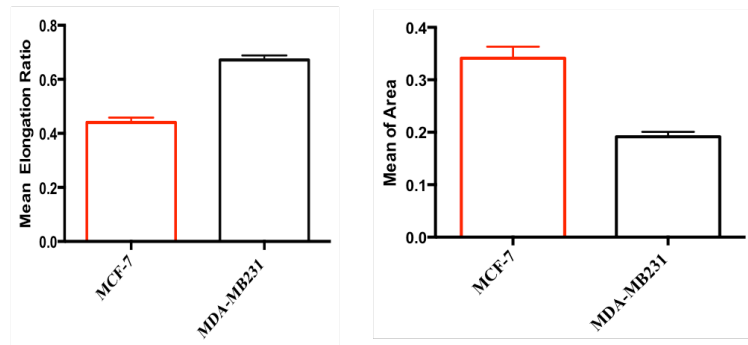
### **3.2.5. Growth factor mediated cell morphology changes**

Both the MDA-MB-231 and MCF-7 cell lines express HGF and EGF receptors (Figure 3.4 and 3.5) and demonstrate a biochemical response to stimulation with the appropriate ligand. However, the morphological response to HGF or EGF stimulation has not been reported. MCF-7 cells stimulated with HGF for 15 minutes exhibit significantly higher cell elongation, when compared to serum starved cells (Figure 3.11). MCF-7 cells stimulated with EGF for 15 minutes also trended towards increased elongation, but this did not reach significance in this data set (Figure 3.11). In contrast to MCF-7 cells, EGF stimulation of MDA-MB-231 cells resulted in a significant decrease in elongation (Figure 3.12), whilst cells stimulated with HGF revealed no difference compared to serum starved cells (Figure 3.12).

A)



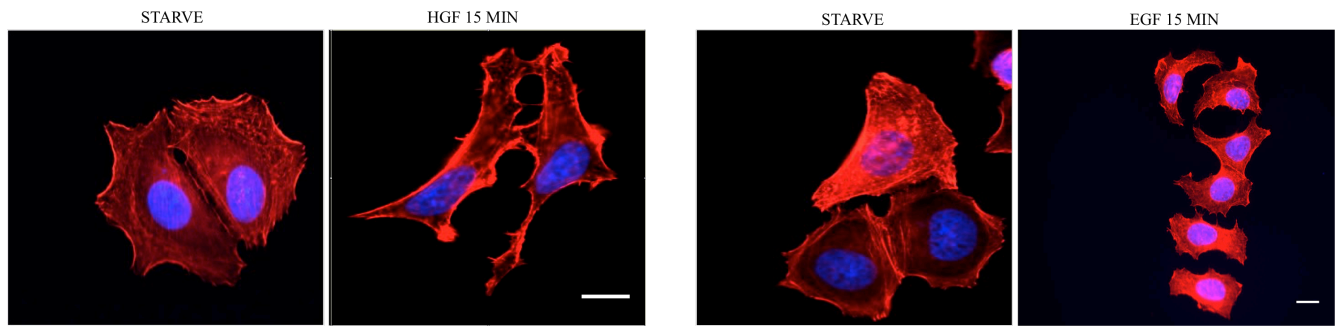
B)



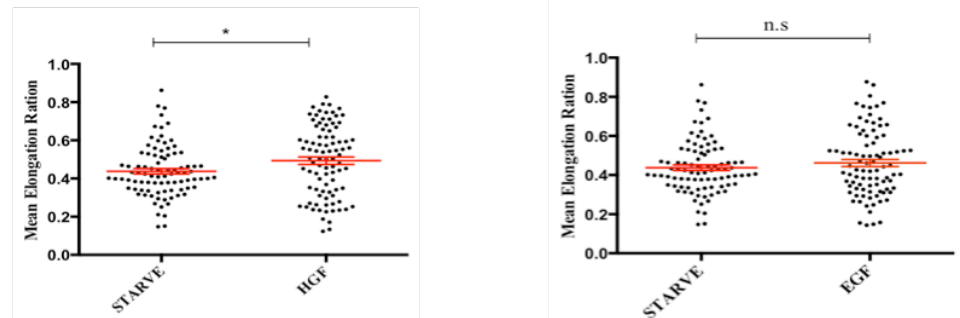
**Figure 3.8: MCF-7 and MDA-MB-231 cell morphology.** Both cell lines were examined by immunofluorescence staining to characterise their cellular morphology. (A) MCF-7 or MDA-MB-231 cells were seeded on glass coverslips and actin stained using TRITC conjugated phalloidin (Red) and stained with DAPI (blue). (B) Cell shape analysis of breast cancer cell lines. The cell elongation or areas were measured using Image J. The standard error of the mean was calculated for 90 cells in three independent experiments. Bars represents the mean value of  $\pm$  S.E.M. Scale bar = 10 $\mu$ m.



A)

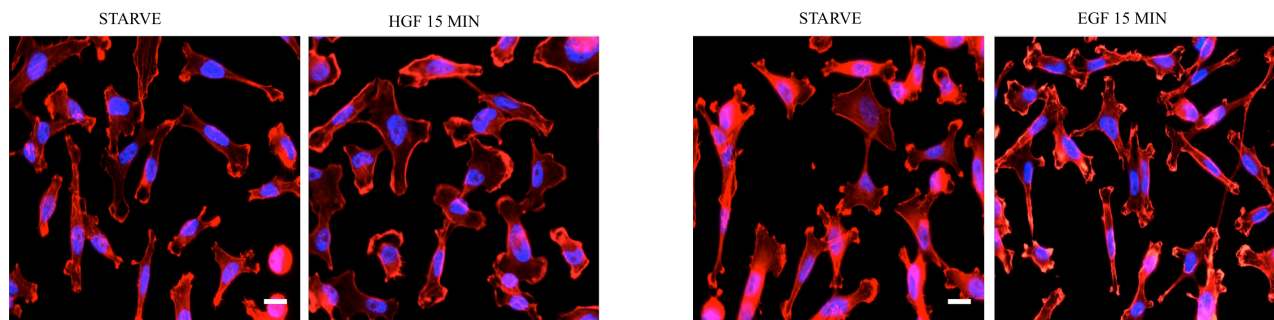


B)

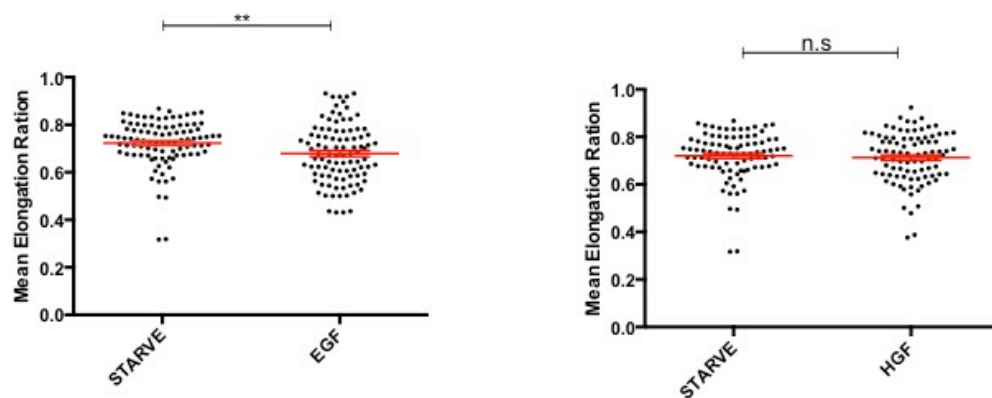


**Figure 3.9: Changes in cell morphology in MCF-7 cells downstream HGF but not EGF stimulation.** A) MCF-7 cells were seeded onto glass coverslips and serum starved for 24 hours. HGF (10 ng/mL) or EGF (100 ng/mL) were added for 15 min, then fixed and stained for F-actin (red) and DAPI (blue). Images were taken using an Olympus IX71 inverted time-lapse microscope with 40x magnification. Shape analysis was performed on the cells using Image J. The standard error of the mean was calculated for 90 cells in three independent experiments to determine the elongation ratio. B) Statistical significance was calculated using *t*-test, \* $P < 0.05$ , n.s.=not significant. Bars represent the mean  $\pm$  S.E.M. Scale bar = 10 $\mu$ m.

A)



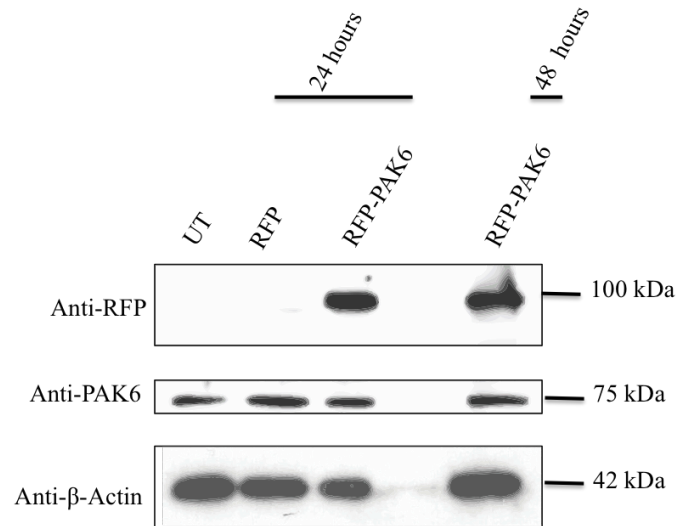
B)



**Figure 3.10: Changes in cell morphology in MDA-MB-231 cells downstream EGF but not HGF stimulation.** A) MDA-MB-231 cells were seeded onto glass Coverslips and serum starved 24 hours. HGF (10ng/mL) or EGF (100ng/mL) were added for 15 min, then fixed and stained for F-actin (red) and DAPI (blue). Images were taken using an Olympus IX71 inverted time-lapse microscope with 40x magnification. Shape analysis was performed on the cells using Image J. The standard error of the mean was calculated for 90 cells in three independent experiments to determine the elongation ratio. B) Statistical significance was calculated using *t*-test, \*\* $P < 0.005$ , n.s.=not significant. Bars represent the mean  $\pm$  S.E.M. Scale bar = 10 $\mu$ m.

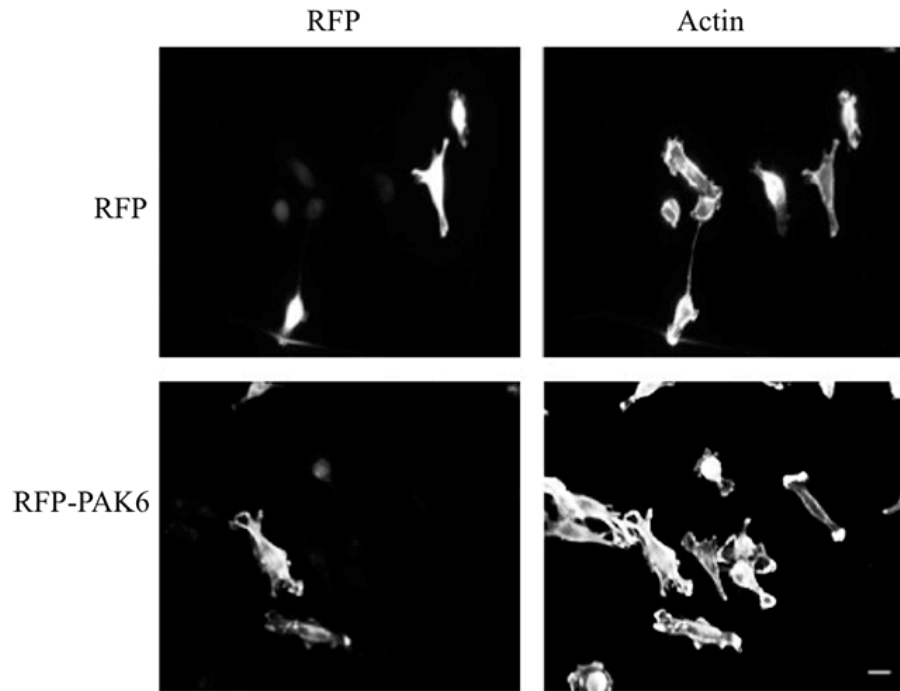
### **3.2.6. PAK6 induces cell elongation in MCF-7 cells but not in MDA-MB-231 cells**

Previous work in the Wells Lab had established that overexpression of PAK6 can induce cell shape changes in prostate cancer cells. The initial aim of this project was to establish whether PAK6 overexpression could also regulate the cell shape of selected breast cancer cell lines. Given that neither HGF nor EGF treatment of the cells lines delivered a robust change in the level of phosphorylated PAK6 (Figure 3.6-3.9), it was decided that further experiments should investigate the impact of PAK6 overexpression in cells under basal conditions. RFP-PAK6 was initially expressed in MDA-MB-231 cells, and the full-length protein was detected by western blot to confirm that expression could be maintained for at least 48 hours, post transfection (Figure 3.13). Overexpression of wild type full-length PAK6 (referred to as PAK6 in all figures) in MDA-MB-231 did not significantly affect cell shape or cell area (Figure 3.14). This result is consistent with previous work that reported that the overexpression of wild type PAK4 in MDCK cells had no effect on cell morphology (Wells et al., 2002). Moreover, cell shape changes were not observed when wild type PAK4 was overexpressed in C2C12 mouse myoblast cells or NIH3T3 mouse embryo fibroblast cells (Qu et al., 2001, Dan et al., 2001b). However, MCF-7 cells expressing RFP-PAK6 shows significantly higher elongation when compared to control cells expressing RFP alone or untransfected cells (Figure 3.15). This result is consistent with previous work from the lab which demonstrated that overexpression of PAK6 can induce cell elongation in the DU145 prostate cancer cells (Fram et al., 2014).

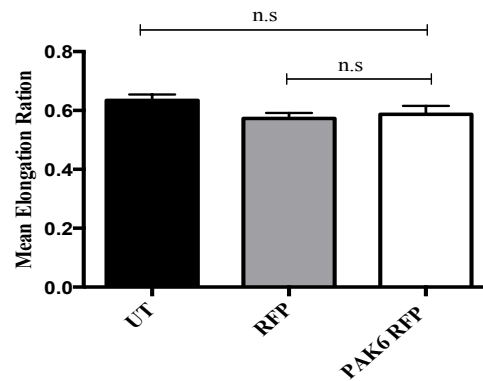


**Figure 3.11: PAK6 overexpression in MDA-MB-231 cells.** MDA-MB-231 cells were transfected with RFP or RFP-PAK6 for 24 hours or 48 hours. Cell lysates were prepared immunoblotted for RFP expression using anti-RFP antibody and for PAK6 using anti-PAK6 antibody. The molecular weight for PAK6 is 75 kDa and  $\beta$ -actin 42 kDa. Lysates were also immunoblotted for  $\beta$ -actin to provide a loading control. The blots shown are representative of three independent experiments.

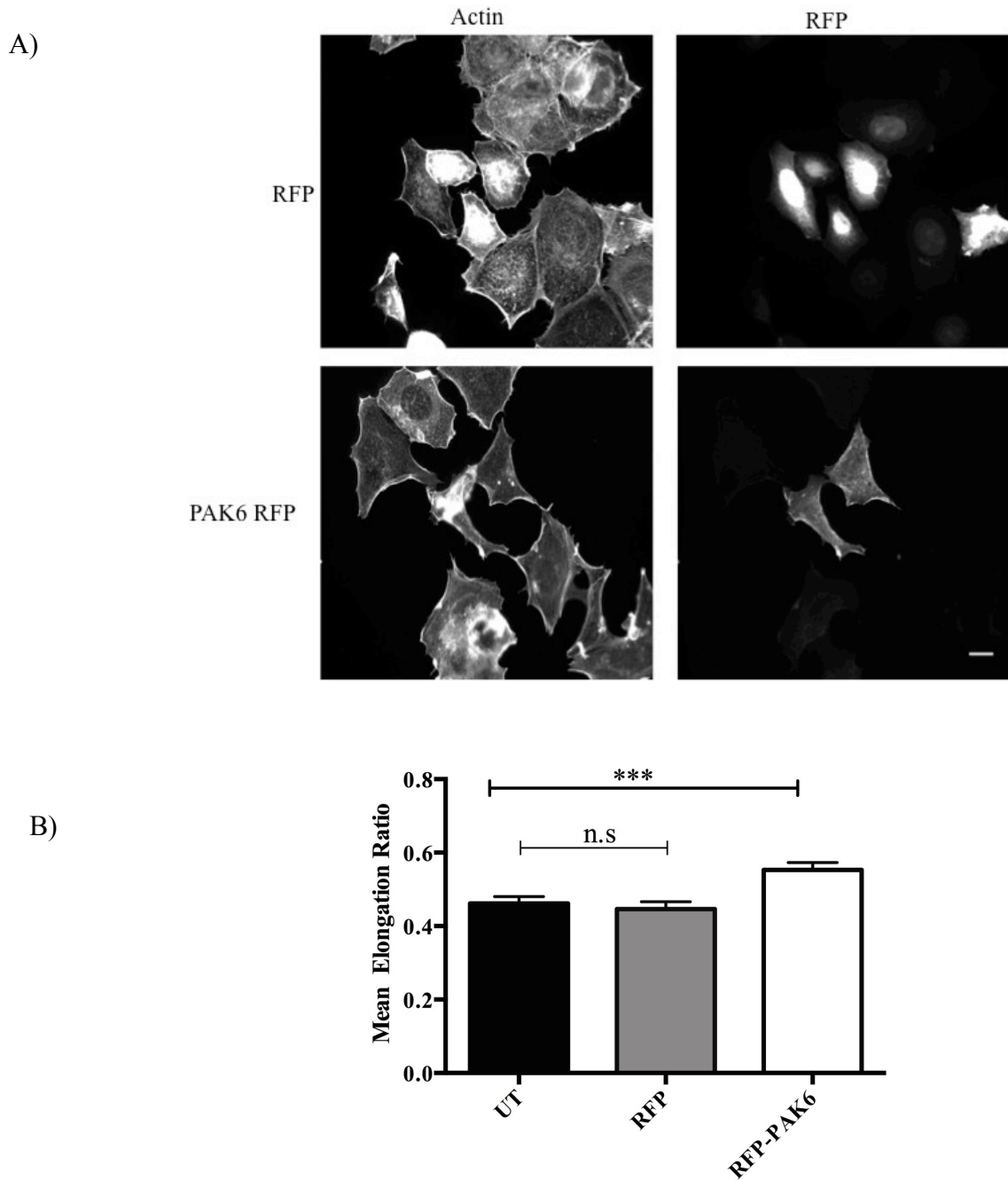
A)



B)



**Figure 3.12: Overexpression wild type PAK6 does not induce changes in cell morphology in MDA-MB-231 cells.** A) MDA-MB-231 cells were seeded onto glass coverslips and transfected with RFP-PAK6 or RFP alone using lipofectamine 2000. Untransfected (UT) or RFP cells were used as a control cells. Cells were then fixed after 24 hours and stain with TRITC-phalloidin for actin or DAPI. Shape analysis was performed on the cells using Image J to determine the elongation. B) The standard error of the mean was calculated for 90 cells in three independent experiments. Statistical significance was determined by using *t*-test, n.s.=not significant. Bars represent the mean  $\pm$  S.E.M. Scale bar = 10 $\mu$ m.



**Figure 3.13: overexpression wild type PAK6 induces changes in cell morphology in MCF-7 cells.** A) MCF-7 cells were seeded onto glass coverslips and transfected with RFP-PAK6 and RFP alone using lipofectamine. Untransfected cells and RFP were used as a control. Cells were then fixed after 24 hours and stained with TRITC-phalloidin (actin) or DAPI. Shape analysis was performed on the cells using Image J to determine the elongation. B) The standard error of the mean was calculated for 90 cells in three independent experiments. Statistical analysis was calculated using *t*-test, \*\*\* $P < 0.0005$  n.s.=not significant. Bars represent the mean  $\pm$  S.E.M. Scale bar  $10\mu\text{m}$ .

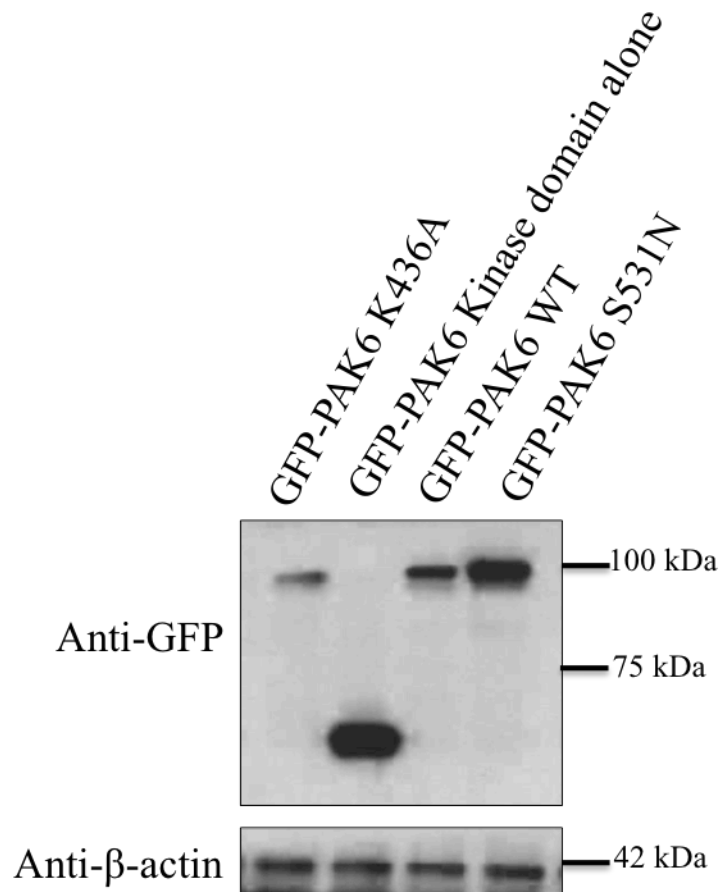
### **3.2.7. Overexpressing active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells**

Since the morphological response of MCF-7 cells phenocopies the morphological effect that exhibited by DU145 prostate cells (Fram et al., 2014), which has already been extensively explored, this project was directed to better understand the role of PAK6 in the non-colony forming MDA-MB-231 cells. It has previously been shown that whilst wild type PAK4 did not induce cell morphology changes, the overexpression of activated PAK4 could induce cell rounding (Wells, 2002). Thus, the overexpression of activated PAK6 was tested here and a number of PAK6 mutant constructs were used (Fram et al., 2014). GFP-PAK6 (K436A) has a lysine to alanine mutation at amino acid 436 in the activation loop, and the mutation of this residue is proposed to inhibit ATP binding and thereby inhibit PAK6 kinase activity (Schrantz et al., 2004), thus rendering a kinase dead PAK6. GFP-PAK6 (S531N) has a serine to asparagine mutation at amino acid 531 in the catalytic loop to render PAK6 constitutively activated. Mutation of this residue is thought to stabilize the catalytic loop region in the kinase domain of PAK6 and is analogous to the PAK4 activation site (S445N) (Schrantz et al., 2004, Qu et al., 2001) and the kinase domain alone located between amino acids 368–682.

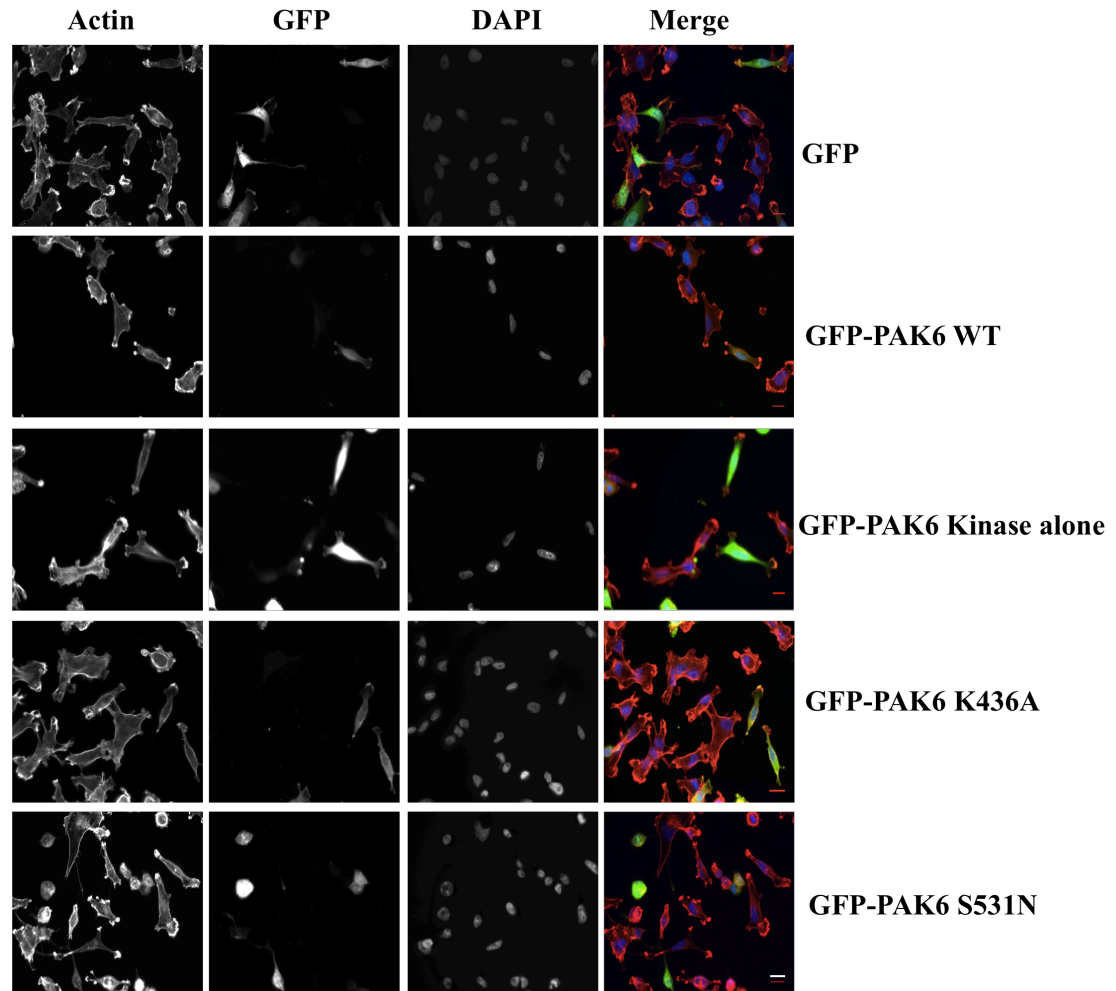
Initially, the PAK6 kinase domain alone, kinase dead or active forms were transfected into MDA-MB-231 cells to confirm the correct expression size of PAK6 constructs. Western blot analysis of transfected cell lysates shows that the exogenous expression of PAK6 kinase dead or kinase active PAK6 was detected at the correct size for full-length proteins (Figure 3.16), whilst PAK6 kinase domain alone was detected at the correct size for a truncated protein (Figure 3.16). I acknowledge there is a variation in the expression of wild type PAK6 and active PAK6. Subsequently, wild type PAK6, Active PAK6 (S531N), PAK6 kinase domain alone or PAK6 kinase dead (K436A) were transfected into MDA-MB-231 cells to monitor the effect on cell morphology. Twenty-four hours following transfection, cells expressing wild type PAK6, kinase dead PAK6 or PAK6 kinase domain alone expressing cells were not significantly different in morphology when compared to cells expressing GFP alone (Figure 3.17).

Interestingly, active PAK6 (S531N) expressing cells were significantly more rounded cells compared with cells expressing the GFP alone (Figure 3.18). It should be noted that when I conducted the shape analysis I excluded highly expressed cells from the analysis, which is more than 2x brighter than WT signal. Moreover, the morphological effect of PAK6 overexpression can be recapitulated when cells are seeded on collagen and fibronectin, suggesting that the phenotype is not matrix dependent (Figure 3.19 and Figure 3.20).

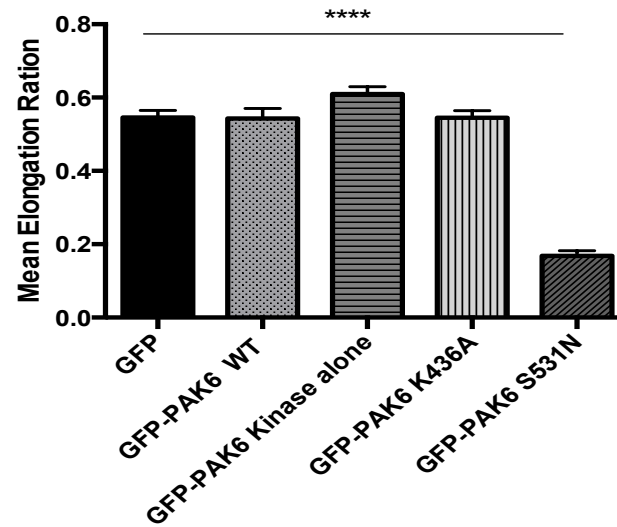




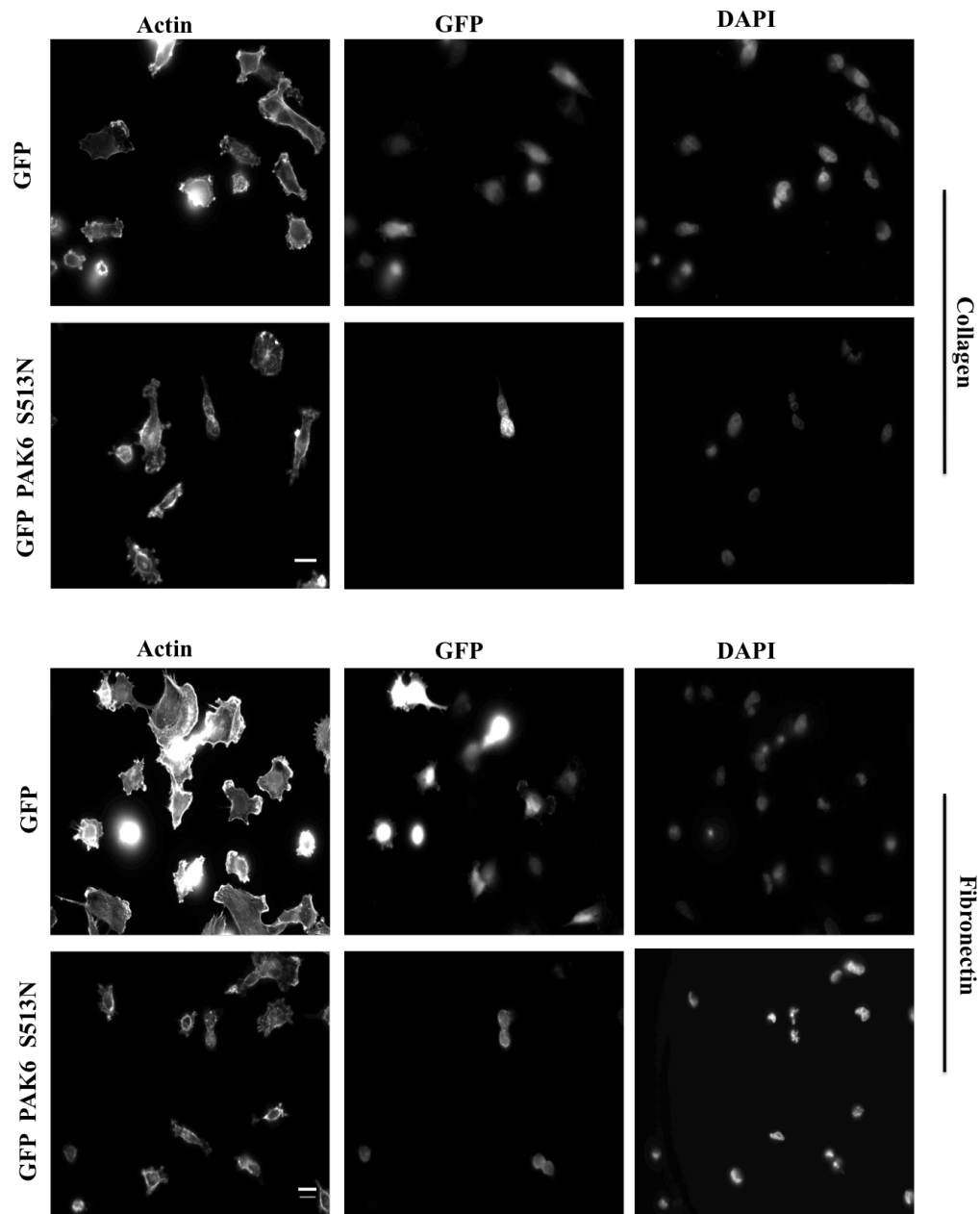
**Figure 3.14: Overexpressing PAK6 mutants in MDA-MB-231 cell.** MDA-MB-231 cells were transfected with PAK6 kinase domain alone, active PAK6 (S531N) or PAK6 dead (K436A). After 24 hours, cells lysates immunoblotted with anti-GFP or anti  $\beta$ -actin antibody, which acted as loading control.



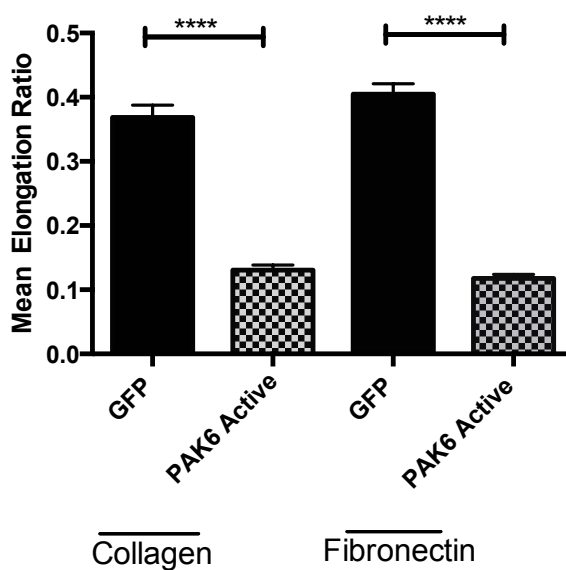
**Figure 3.15: Overexpressing the active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells.** MDA-MB-231 cells were seeded on glass coverslips and transfected with GFP control vector or GFP-PAK6 mutants as indicated. After 24 hours, cells were fixed and stained with TRITC-phalloidin for actin or DAPI. 90 cells over three independent experiments were analysed. Scale bar = 10 $\mu$ m.



**Figure 3.16: Overexpressing the active form of PAK6 (S531N) induces cell rounding in MDA-MB-231 cells.** Shape analysis was performed on the cells using Image J to determine the elongation ratio. 90 cells over three independent experiments were analysed. Statistical significance compared with GFP expressing control cells was calculated using *t*-test, \*\*\*\* $P < 0.0001$ . Bars represent the mean  $\pm$  S.E.M.



**Figure 3.17: Overexpressing the active form of PAK6 (S531N) in MDA-MB-231 cells seeded on collagen and fibronectin.** MDA-MB-231 cells were seeded on collagen and fibronectin and transfected with GFP or GFP Active PAK6 (S531N). After 24 hours, cells were then fixed and stained with TRIC-phalloidin for actin and DAPI. Shape analysis was performed on the cells using Image J to determine the elongation ratio as indicated. Representative image are shown three independent experiments.



**Figure 3.18: Overexpressing the active form of PAK6 (S531N) in MDA-MB-231 cells seeded on collagen and fibronectin.** Quantification results represents three independent experiments. Bars are standard error of mean value. Statistical significance compared with GFP control cells was calculated using *t*-test, \*\*\*\* $P < 0.0001$ .

### **3.3.Discussion:**

In this chapter, the PAK6 protein expression levels in breast cancer cell lines have been investigated. Regarding PAK expression, there has been widely research into how they contribute to disease progression and cancer (King et al., 2014). However, there are very few studies on PAK6 expression levels and in particular in breast cancer. A panel of breast cell lines were therefore investigated for PAK6 protein expression, in order to determine the most appropriate cell lines to use going forward within the project investigation and test whether the level of expression is correlated with invasive potential. Northern blot analysis has shown that PAK6 is expressed in the testis and prostate tissues (Yang et al., 2001). In addition, it is reported that PAK6 expressed in two breast cancer cell lines MDA-MB-231 and MCF-7 (Kaur et al., 2008a). Previous work from our laboratory, demonstrated links between PAK6 expression and cancer cell invasiveness in the prostate cancer cell line DU145, with overexpression of PAK6 leading to colony dissemination (Fram et al., 2014). In my study, PAK6 was found to be expressed in both single cells and colony forming breast cancer cell lines MDA-MB-231 and MCF-7 respectively, with higher expression trending in invasive breast cancer cells, although these results did not reach significance. Overall, the results indicate that PAK6 plays roles in breast cancer cells in addition to those previously assigned to function at cell:cell junctions (Fram et al., 2014).

To facilitate PAK6 studies, efforts were made to develop an in-house-PAK6 antibody. However, although this in-house antibody could specifically detect overexpressed PAK6, the antibody did not recognize the expression of endogenous PAK6 at the correct size. The antibody was not used further as a new commercial antibody became available that did detect endogenous PAK6 at correct size and this reagent was used for the remainder of the project.

Little was known about the regulation of PAK6 at the start of this project. The phosphorylation of PAK6 on a specific serine residue Ser560 was thought a sign of its activation (Kaur et al., 2005). PAK6 had also been shown to be activated downstream HGF in prostate cancer cells by other members of our laboratory (Fram et al., 2014). Therefore, the level of PAK6 autophosphorylation following growth factor stimulation (EGF and HGF) was investigated in breast cancer cells. Two breast cancer cell lines were selected, MDA-

MB-231 and MCF-7; both express PAK6, and represent single cell and colony forming cells respectively. In both tested cell lines, it is known that the activation of the receptors for HGF and EGF results in the phosphorylation of many downstream effectors. MEK-dependent phosphorylation of the MAP kinases ERK1 and ERK2 is the best-studied pathway, and this was used as a positive control for HGF or EGF stimulation. Both MDA-MB-231 cells and MCF-7 cells showed an increase in the phosphorylation of ERK, with the response being evident after only 5 minutes. This observation confirmed that HGF/c-Met and EGF/EGFR signalling is highly active within these cell lines. However, with regards to PAK6, the results suggest that PAK6 autophosphorylation levels do not increase upon HGF or EGF stimulation in either MDA-MB-231 or MCF-7 cells (Figure 3.6-3.9). This could be due to the efficiency of the phospho-serine 560 antibody, or it is possible that PAK6 is phosphorylated downstream of HGF or EGF on residues other than serine 560, and this has been shown for other PAK family members including PAK1 where multiple autophosphorylation sites have been observed (Chong et al., 2001). In addition, it is known that whilst the activation of PAK6 via MKK6 requires serine 560 phosphorylation, PAK6 is also directly activated by phosphorylation on tyrosine 566 by MKK6 (Kaur et al., 2005). This supports a hypothesis that phosphorylation of other residues on PAK6 may be important for PAK6 activation. This could be investigated more by designing in-house antibodies to different serine, threonine or tyrosine sites on PAK6. Furthermore, recent publications have suggested that group II PAKs may in fact be constitutively phosphorylated at this site and that their regulation is controlled either by the release of an autoinhibitory domain, or a pseudosubstrate region within the N-terminal (Baskaran et al., 2012, Ha et al., 2012). Thus, from these studies it was concluded that at least in these two breast cancer cell lines, HGF/EGF might not play a prominent role in PAK6 activation via S560 phosphorylation.

Generally, when PAK family proteins are overexpressed in cells, morphological changes are only detected when activated mutants are expressed (King et al., 2014). Indeed, wild type PAK4 overexpression demonstrates no effects on MDCK cell morphology (Wells, 2002). In addition to PAK4, overexpression of wild type PAK1 has no affect on cell morphology, while the constitutively active forms of PAK1 induces loss of intracellular stress fibres (Manser et al., 1997). However, previous work in our laboratory has indicated that overexpression of wild type PAK6 could induce cell morphology changes, with overexpression of wild type PAK6 inducing cell elongation in DU145 prostate cancer cell lines (Fram et al., 2014). Here, in MCF-7 cells, the overexpression of wild type PAK6 was again found to induce cell elongation (Figure 3.15). The elongation phenotype observed when wild type PAK6 is overexpressed in MCF-7 cells is likely due to a decrease in cell:cell adhesion. This has not been reported before for MCF-7 cells and suggests that PAK6 may also play a role in cell:cell adhesion beyond DU145 cells. Thus further investigation of PAK6 function in the cell:cell adhesion of MCF-7 cells is warranted. The ability of wild type PAK6 expressing cells to prompt a morphological change could be related to the phosphorylation status of wild type PAK6 and thus activity levels. Wild type PAK6 may be autophosphorylated under basal conditions, as has been speculated in the literature for group II PAKs (Pandey et al., 2002, Xu et al., 2010, Fram et al., 2014).

In contrast, the overexpression of wild type PAK6 in MDA-MB-231 cells does not induce significant differences in cell shape. However, it might be that for MDA-MB-231 cells, the PAK6 needs to be an activated version. Indeed, the majority of PAK induced cell shape changes reported previously have been detected when constitutively active forms of PAK1, 2 and 4 were used (Wells, 2002, Manser et al., 1997, Qu et al., 2001, Zeng et al., 2000). Consistent with this hypothesis, cell shape does not change when wild type PAK4 was overexpressed in C2C12 mouse myoblast cells or NIH3T3 mouse embryo fibroblast cells (Dan et al., 2001b, Qu et al., 2001). The lack of cell shape change might indicate that PAK6 has a different functional role in MDA-MB-231 cells compared to MCF-7 cells; indeed, MDA-MB-231 cells do not form cell:cell adhesions. Results reported here found that the overexpression of the active PAK6 (S531N) induces cell rounding, whereas overexpression of PAK6 (K436A) or the PAK6 kinase domain alone had no impact on cell shape (Figure 3.18).



The PAK6 kinase domain alone construct is thought to be constitutively activate (Yang et al., 2001). However, in this assay it did not induce a rounding phenotype (Figure 3.18). This observation may be due to incorrect localisation of the protein indeed; PAK6 kinase domain alone was not routinely detected at the cell periphery, but rather exhibited a diffuse cytoplasmic localisation. The cell rounding morphology induced by active PAK6 (S531N) is reminiscent of that induced by the expression of activated PAK4 in mouse embryo fibroblast cells, C2C12 mouse myoblast cells or NIH3T3 cells (Qu et al., 2001, Dan et al., 2001b), and it was suggested that cells may become rounded due to loss of adhesion. The cell rounding phenotype observed following expression of active PAK6 in MDA-MB-231 cells might be due to a number of different mechanisms. It could also be due to a defect in cell spreading. Indeed, studies have shown that Rat1 fibroblast cells expressing active PAK4 exhibited a diminished ability in cell spreading on a fibronectin-coated substratum (Qu et al., 2001). Furthermore, evidence suggests that PAK family member play a role in cell-substratum adhesion turnover (Manser et al., 1997, Wells, 2002, Whale, 2011). Alternatively, cell rounding may be the result of increased actomyosin contractility (Sahai and Marshall, 2003).

Work in this chapter has confirmed that there was a high level of PAK6 expression in MDA-MB-231 cells. Moreover, the overexpression of activated PAK6 was able to induce cell shape changes in these cells. These data support further investigation of the role of PAK6 in MDA-MB-231 cells.

### 3.4.Future Work

Based on the activation of PAK6 data in this chapter, it would be interesting to test the phosphorylation by performing *in vitro* kinase assay by overexpressed PAK6 and immunopurified from stimulated and unstimulated cell lysates. Moreover, another PAK6 phosphorylation antibody could be tested. Breast cancer PAK6 mutations are reported in the COSMIC database, and it would be interesting to see if these mutations also impact on cell morphology; particularly mutations in the kinase domain for example, this PAK6 mutation:

[http://cancer.sanger.ac.uk/cosmic/gene/samples?coords=AA%3AAA&src=gene&hn=carcinoma&end=682&ln=PAK6&ss=NS&all\\_data=&sn=breast&sh=basal\\_\(triple-negative\)\\_carcinoma&seqlen=682&id=346&start=1#complete](http://cancer.sanger.ac.uk/cosmic/gene/samples?coords=AA%3AAA&src=gene&hn=carcinoma&end=682&ln=PAK6&ss=NS&all_data=&sn=breast&sh=basal_(triple-negative)_carcinoma&seqlen=682&id=346&start=1#complete).

**Chapter 4:**  
**Characterisation of cell morphology in PAK6**  
**modulated cells**

## **4. Chapter 4: Characterisation of cell morphology in PAK6 modulated cells**

### **4.1. Introduction**

Cell migration and invasion play a key role in metastasis and are considered hallmarks of cancer. To metastasize, tumour cells must move through and across boundaries, and this process requires cell motility, remodelling of cell-cell contacts and additional interactions with the extra cellular matrix (Fidler, 1999). Tumour cells can move as an individual or as a collective group (Wolf et al., 2007). Others have demonstrated that individual tumour cells can display two different types of movement: an amoeboid mode, with a rounded morphology and no obvious polarity, or alternatively a mesenchymal mode, characterised by an elongated morphology that requires extracellular proteolysis which occurs at cellular protrusions (Friedl and Wolf, 2003). Previous studies have shown that amoeboid or mesenchymal modes of movement are distinguished and regulated by different signalling pathways (Sahai and Marshall, 2003). The amoeboid phenotype is characterised by high levels of actomyosin contractility driven by ROCK (Wilkinson et al., 2005a), whereas the mesenchymal mode of movement requires extracellular proteolysis (Sahai and Marshall, 2003).

The processes of cell polarity, cell shape, cell adhesion, motility and/or cytokinesis each require reorganization of the actin cytoskeleton. Cell motility is determined by the cycles of actin polymerization, cell adhesion and actomyosin contractility (Sahai et al., 2007). The purification of motile cells from living tumours has shown that the actin polymerization machinery is more active in these cells when compared with non-motile cancer cells extracted from the same tumour. Therefore, it is important to identify the mechanisms by which cytoskeletal regulatory proteins control polymerized actin structures. The RhoGTPase family has been implicated in multiple cellular responses, including the regulation of the actin cytoskeleton, cell adhesion, motility, the activation of signal transduction pathways, the control of cell proliferation, oncogenic transformation and/or invasiveness (Vega and Ridley, 2008, Yilmaz and Christofori, 2010).

The best characterised RhoGTPases are Rho, Rac1 and Cdc42, which have been shown to regulate cell shape, migration and invasion primarily by actin polymerization/depolymerization and the branching of actin filaments. Rac1 drives motility by promoting lamellipodia formation (Ridley, 1992b), while RhoA signalling occurs via Rock I and II kinases, and subsequent phosphorylation and activation of myosin light chain 2 (MLC2), thereby promoting the formation of actin stress fibres and the generation of the actomyosin contractile force required for cell movement (Riento and Ridley, 2003, Sanz-Moreno et al., 2008).

The most well-characterised downstream effectors of Cdc42 and Rac are the PAK family of protein kinases (Bishop and Hall, 2000). However, whilst Group I PAKs have been extensively studied, PAK6 is relatively uncharacterised. There are no studies on the morphological impact of PAK6 knockdown in breast cancer cells.

Traditionally, cell migration studies have focused on 2D migration assays however, it is becoming evident that 3D assays provide a better and more relevant system is better to study cell invasion (Friedl and Wolf, 2003, Wolf et al., 2003). Indeed, it has been shown that cells can demonstrate phenotypic differences in a 2D environment, when compared to 3D studies. Therefore, to complement 2D experiments, many recent studies have employed 3D assays to investigate invasion in an environment that more closely resembles that seen *in vivo* (Blacher et al., 2014). The role of PAK6 in 3D cell invasion has not been investigated.

In this chapter, the modulation of PAK6 expression levels will be used to further characterise the role of this protein in cell morphology and invasion.

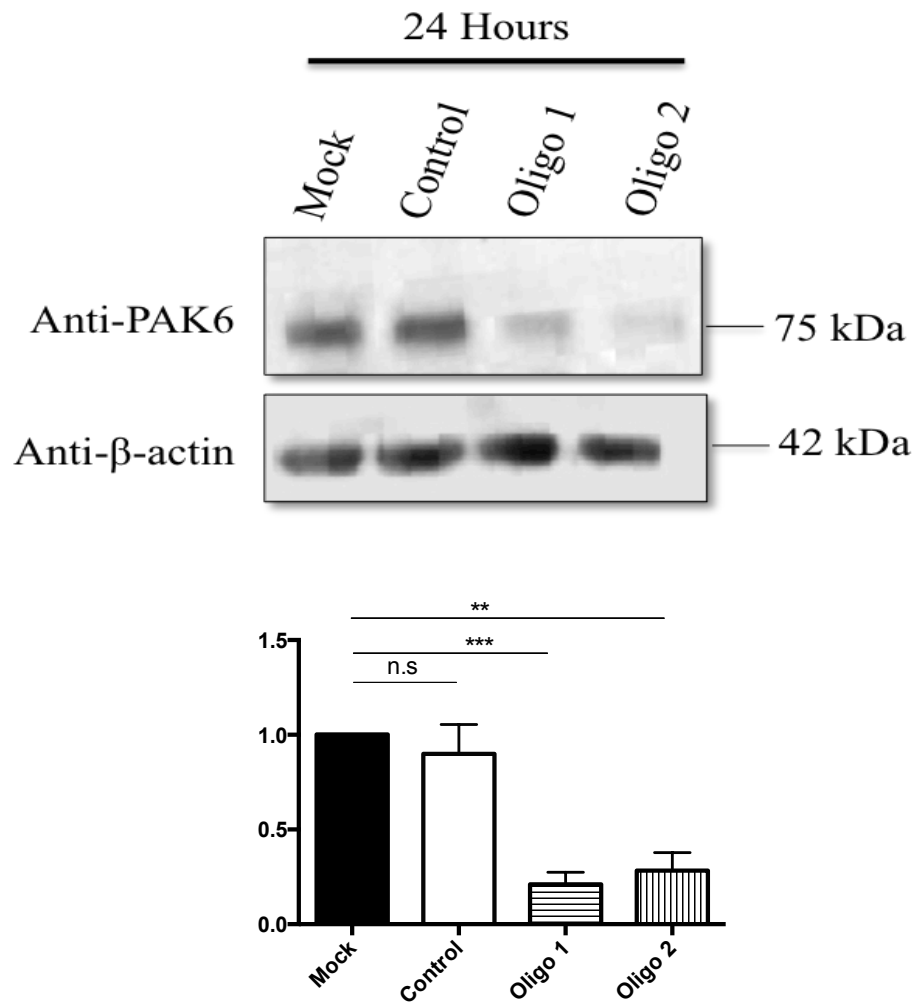
## **4.2. Results:**

### **4.2.1. PAK6 knockdown in MDA-MB-231 cells**

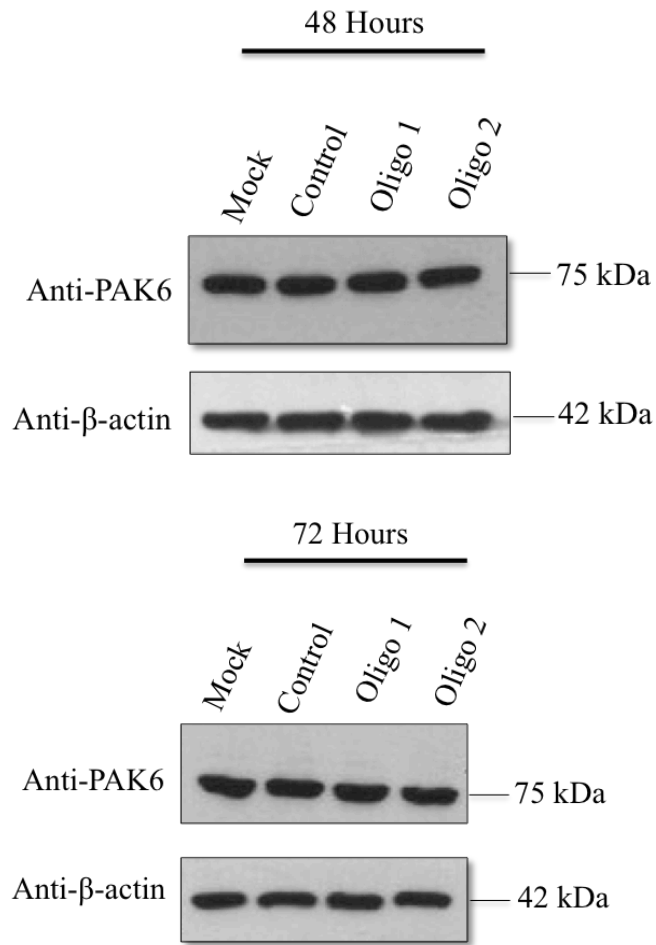
In the previous chapter, the overexpression of activated PAK6 (S531N) was shown to induce cell rounding in MDA-MB-231 cells (Figure 3.17). To further explore the relationship between PAK6 and cell shape, a PAK6 knockdown strategy was employed. Two different PAK6 specific siRNA oligonucleotides were tested in MDA-MB-231 cells, alongside a non-targeting control siRNA or untransfected (mock) cells. The level of PAK6 expression was significantly reduced 24 hours after siRNA transfection when compared with PAK6 levels in cells treated with control siRNA. No significant difference in PAK6 expression levels was detected between untransfected mock and control siRNA-treated MDA-MB-231 cells (Figure 4.1). However, there were no differences in PAK6 expression after 48 and 72 hours (Figure 4.2). Suggesting that the PAK6 depletion is a transient event. Whilst PAK6 knockdown was achieved, this was a challenging process that required optimization of the siRNA concentrations, the cell seeding densities used and the time point of detection. This could be due to the fact that PAK6 was identified as a ‘difficult to silence’ gene in an siRNA screen where the central region of PAK6 mRNA was found to be highly difficult to target using siRNA knockdown (Bergauer et al., 2009). Additional experiments using a shRNA vector to generate a stable PAK6 knockdown cell line were conducted; however, after the antibiotic selection, it was not possible to achieve significant reductions in PAK6 expression in the selected cell lines.

### **4.2.2. Depletion of PAK6 has no significant impact on MDA-MB-231 cell proliferation**

Previous studies have reported that PAK6 knockdown using siRNA can significantly inhibit *in vivo* and *in vitro* cell growth in prostate cancer cells (Wen et al., 2009). Knockdown of PAK6 using shRNA technology has also demonstrated a significant reduction of cell viability following irradiation, when compared to the control cells expressing normal PAK6 levels in prostate cancer cells (Zhang et al., 2010).



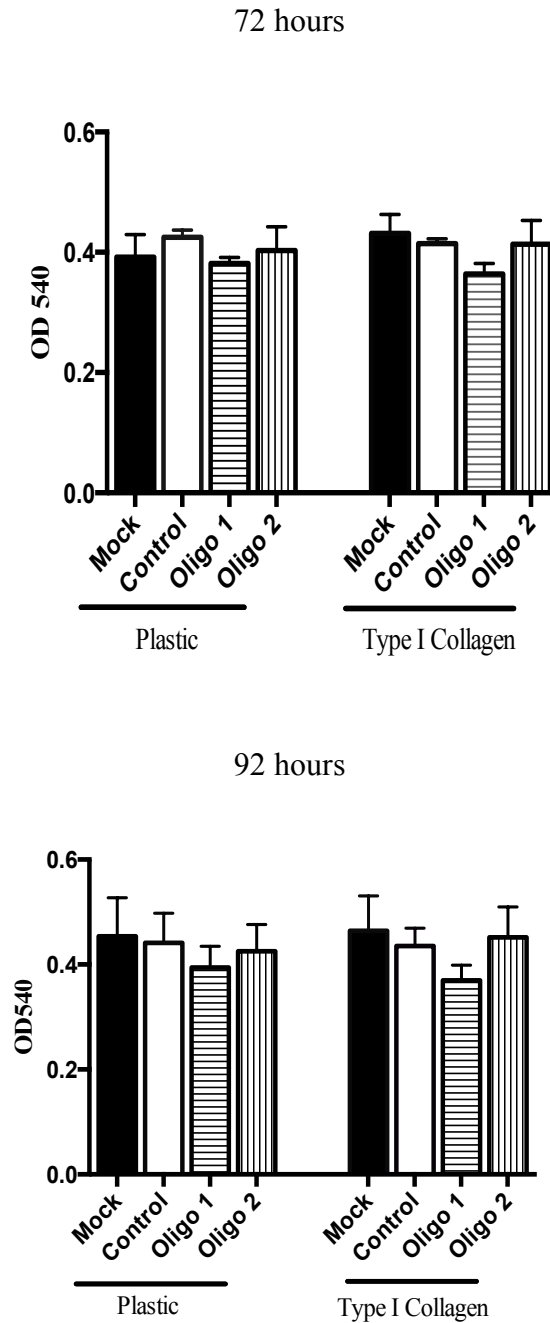
**Figure 4.1: PAK6 knockdown in MDA-MB-231 cells.** MDA-MB-231 cells were seeded and transfected with PAK6 siRNA (Oligo 1 or 2) or control siRNA or untransfected mock. Cells lysed after 24 hours and immunoblotted for PAK6 using a PAK6 specific antibody and for  $\beta$ -actin as a loading control. Quantification of blots representative of three independent experiments, which were quantified using densitometry. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test where \*\* $P=0.0017$ , \*\*\* $P=0.0003$ , n.s.=not significant.



**Figure 4.2: PAK6 knockdown does not effect MDA-MB-231 cells after 48 and 72 hours.** MDA-MB-231 cells were seeded and transfected with PAK6 siRNA (Oligo 1 or 2) or control siRNA or untransfected mock. Cells lysed after 48 and 72 hours and immunoblotted for PAK6 using a PAK6 specific antibody and  $\beta$ -actin as a loading control.



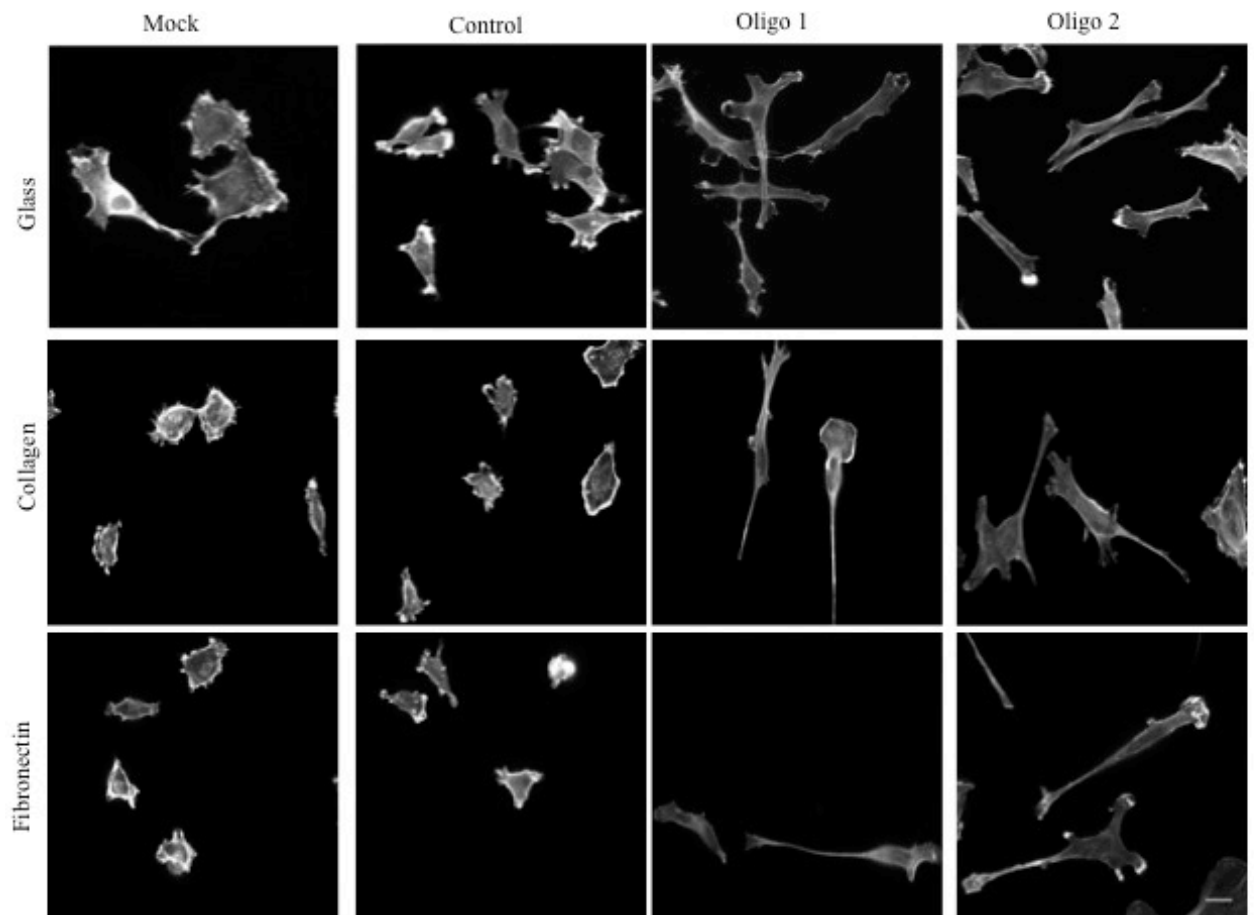
Moreover, PAK6 depletion also reduces the rate of PC3 (prostate) cell proliferation compared to the control cells (Goc et al., 2013). Thus, it was decided to investigate whether MDA-MB-231 cell proliferation was also effected by PAK6 depletion over the time frame of our assay. Control or PAK6 specific siRNA treated cells were replated onto plastic and collagen coated tissue culture plates. Cells were incubated for 72 or 96 hours and an MTT assay conducted to determine the cell proliferation. Different matrixes were used because studies have shown that matrixes can influence the proliferative response of cells in colon cancer (Ohtaka et al., 1996). However, the results suggest that there was no significant difference in cellular proliferation was observed in PAK6 knockdown MDA-MB-231 cells, when compared to control and wild type cells (Figure 4.3). Given that PAK6 knockdown is very transient and is only sustained for 24 hours, it would be optimal to generate a stable knockdown and repeat the proliferation assay.



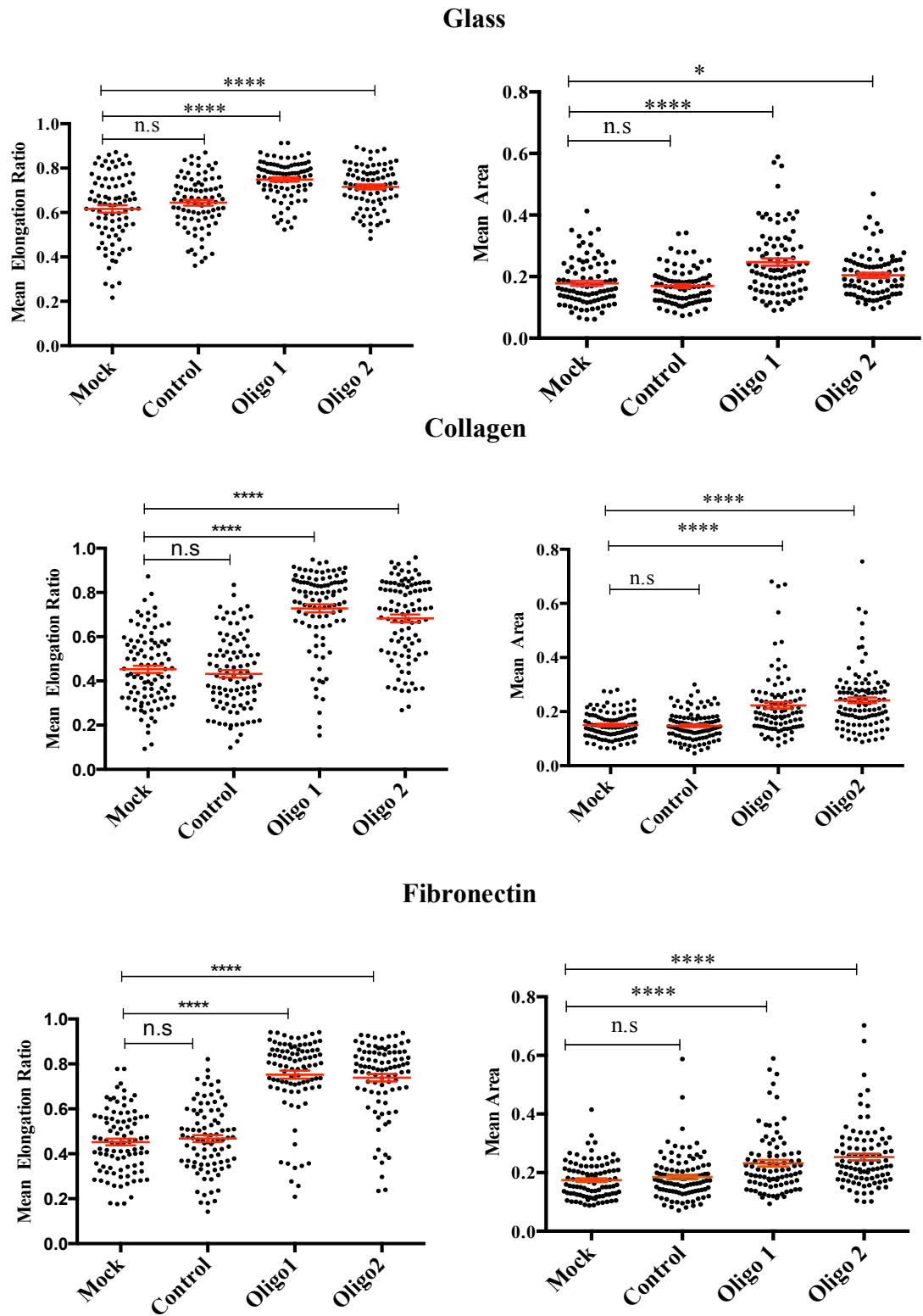
**Figure 4.3: Depletion of PAK6 has no significant impact on MDA-MB-231 cell proliferation.** After siRNA-mediated depletion of PAK6, MDA-MB-231 cells were subjected to an MTT assay to look at the effect of PAK6 knockdown on cellular proliferation. PAK6 knockdown cells were compared with both untransfected (Mock) or control siRNA cells after 72 or 96 hours. There was no significant difference observed in three independent experiments.

#### **4.2.3. PAK6 depletion induces cell elongation**

Optimal PAK6 knockdown was observed 24 hours after siRNA transfection, and morphological characterisation studies of MDA-MB-231 cells after PAK6 depletion were therefore performed at this time point (Figure 4.4). Control or PAK6 knockdown cells were seeded onto glass coverslips, and PAK6 depleted cells appeared to exhibit a more elongated morphology. Following quantification, cells transfected with PAK6 siRNA oligo1 or oligo2 were significantly elongated compared with untransfected (mock) or control cells (Figure 4.5). Furthermore, similar elongated phenotypes were observed when cells depleted of PAK6 were seeded on collagen or fibronectin coated coverslips. This morphological change does not depend on the extracellular matrix composition, as observed with overexpression studies (Figure 3.19). In addition, PAK6 knockdown cells also exhibited a significantly increased cell area (Figure 4.5).



**Figure 4.4: PAK6 knockdown in MDA-MB-231 cells.** MDA-MB-231 cells were subjected to siRNA knockdown of PAK6 using two pre-validated siRNAs. In addition both a control siRNA and mock cells were used in these morphological studies. Cells were seeded onto glass, collagen and fibronectin coated coverslips and stained for actin. Images are representative of three independent experiments. Scale bar = 10 $\mu$ m.



**Figure 4.5: PAK6 knockdown induce cell elongation in MDA-MB-231 cells.** The cell elongation and area were calculated using ImageJ and results quantified. Statistical significance was calculated using ANOVA *Tukey test*. Where  $P^{****} < 0.0001$ ,  $P^* < 0.02$  n.s.=not significant.

#### **4.2.4. LBH inhibitor reduces PAK6 expression**

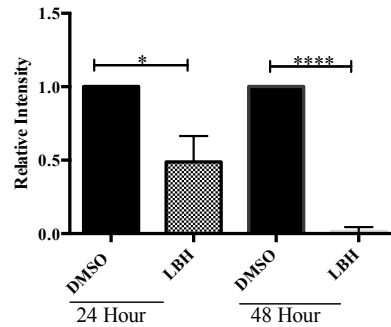
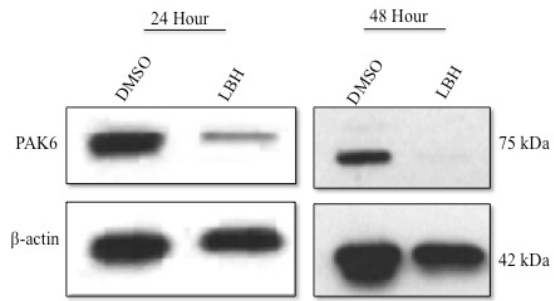
PAK6 siRNA studies were limited to a twenty-four hour knockdown window. However, during these studies, it was reported that the pan-deacetylase inhibitor Panobinostat (LBH) can lead to a decrease in PAK6 expression in a hepatocellular carcinoma cell line (Henrici et al., 2013). This observation raised the possibility that LBH could be used to achieve an extended period of PAK6 depletion in the MDA-MB-231 cells. LBH selectively inhibits histone deacetylase (HDAC) and induces the hyperacetylation of core histone proteins, which may result in the modulation of cell cycle protein expression, cell cycle arrest in G2/M phase and apoptosis. Panobinostat induces growth inhibition and cytotoxicity across, breast, colon, prostate, pancreatic and prostate cancer cell lines and acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), Hodgkin lymphoma (Shao et al., 2014). Many of the genes altered by panobinostat have roles in cell invasion and metastasis involving CDH1, CLDN7, FOSL1, PLAU, STC2, and TGFA (Tate et al., 2012). In addition, HDAC inhibitors stimulate transcription of proapoptotic genes such as Bax, Bak and Apaf1 (Marchion and Münster, 2007). Studies demonstrated that following HDAC inhibitor application, various TNF receptor super family members and their ligands are transcriptionally activated (Johnstone, 2002, Minucci and Pelicci, 2006). Moreover, Panobinostat mediated apoptotic pathways and induced cellular differentiation, this effect is associated with cell-cycle arrest at the G1/S boundary mediated by the retinoblastoma protein (pRb) and related proteins (Gabrielli et al., 2002).

The effect of using the inhibitor on PAK6 expression in the MDA-MB-231 cells was investigated first (Figure 4.6). MDA-MB-231 cells were subjected to the treatment for 24 or 48 hours with 5 nM LBH or with DMSO as a control. The results showed that the expression of PAK6 was significantly decreased after the addition of the LBH and there was some indication that actin expression was also suppressed following LBH treatment (Figure 4.6). Previous studies have shown that triple negative breast cancer cells, but not ER-positive cell lines, treated with LBH have significantly reduced cell proliferation (Tate et al., 2012), and the impact of LBH inhibitor on MDA-MB-231 proliferation was therefore examined. Cells were treated with the LBH inhibitor and incubated for 24, 48

or 72 hours, and an MTT assay was carried out to measure the cell proliferation. The result shows that there was no significant difference in cellular proliferation observed after 24 and 48 hours treatment. A significant decrease in the MDA-MB-231 cell proliferation was however observed after 72 hours post LBH inhibitor treatment (Figure 4.7).

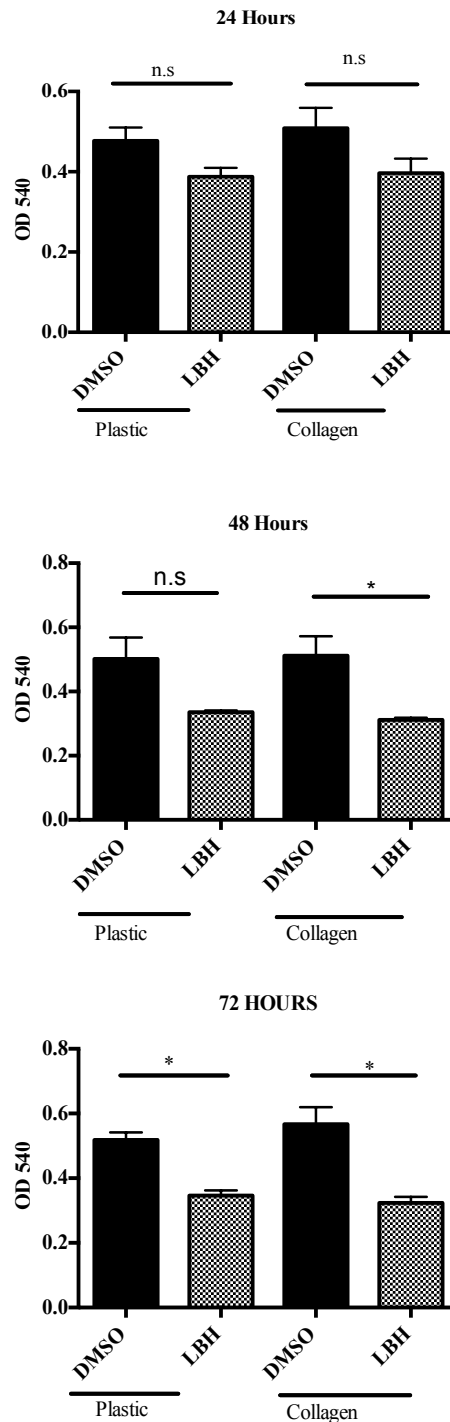
#### **4.2.5. LBH treatment phenocopies PAK6 knockdown cell morphology**

Having established that the LBH treatment depletes PAK6 expression, the effect of adding LBH on MDA-MB-231 cell morphology was investigated. In a time frame 24/48 hours before significantly drop proliferation, the results demonstrated that there was a significant cell elongation upon the LBH inhibitor after 24 or 48hours (Figure 4.8). This result is consistent with the knockdown results (Figure 4.4), which show that the depletion of PAK6 expression leads to cell elongation.

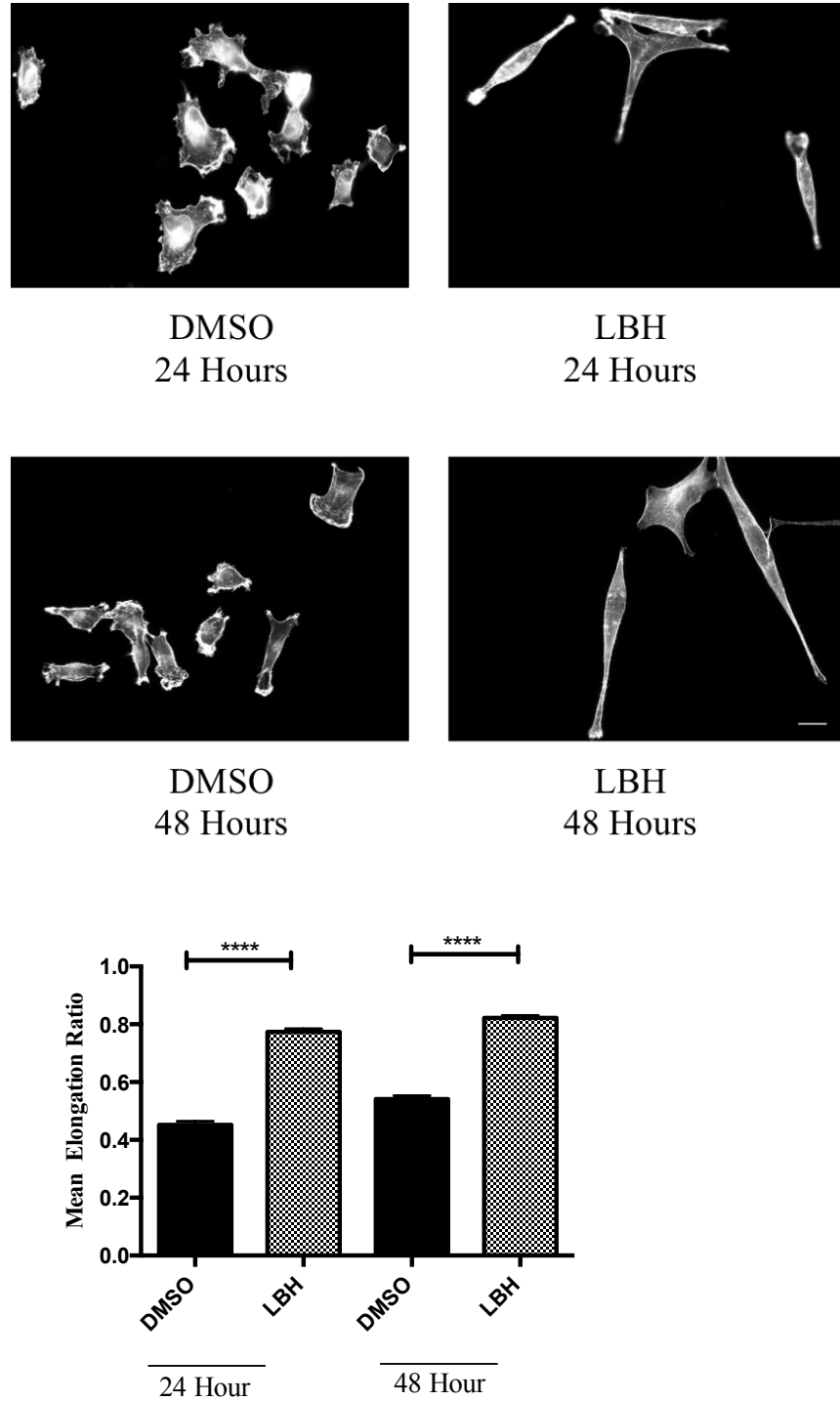


**Figure 4.6: LBH inhibitor reduces PAK6 expression.** The Histone deacetylase inhibitor can reduce the PAK6 expression. MDA-MB-231 cells were treated with 5nM LBH or DMSO as control for 24 or 48 hours. Quantified data represents three independent experiments. Statistical significance was calculated using Student's *t*-test. Where P\*\*\*\*<0.0001, P\*<0.01.





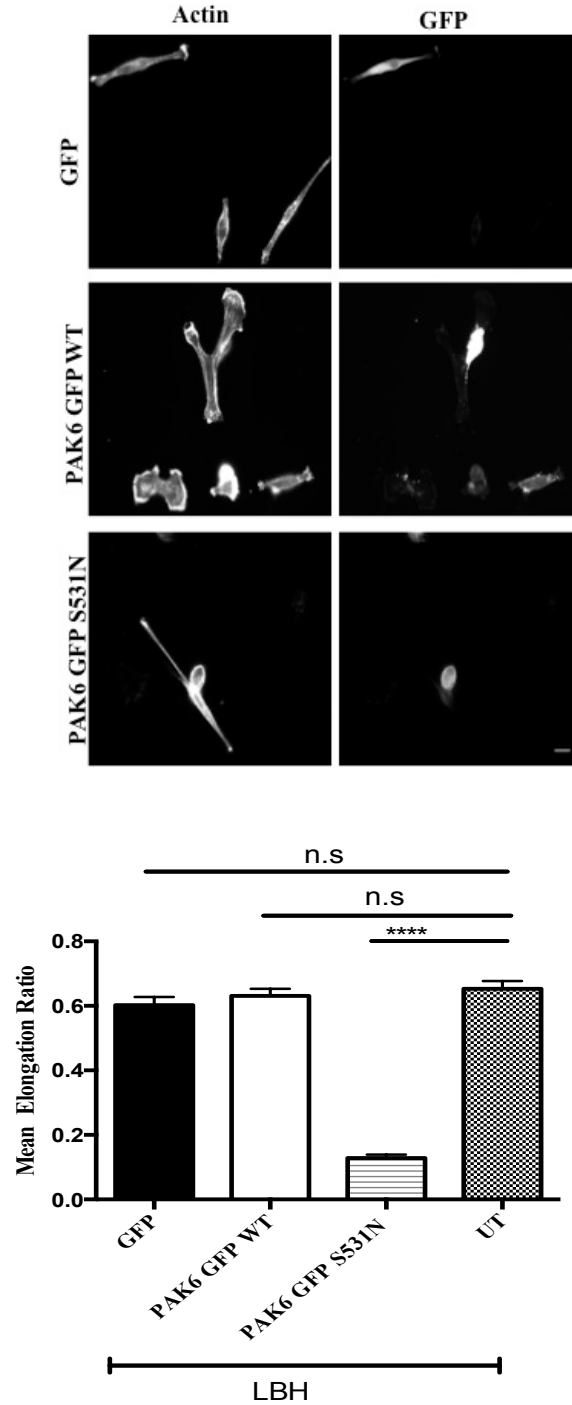
**Figure 4.7: The effect of LBH inhibitor on MDA-MB-231 cell proliferation.** MDA-MB-231 cells were treated with LBH for the indicated times and then subjected to an MTT assay to measure cell proliferation. LBH treated cells were compared with DMSO control cells after 24, 48 and 72 hours. There was no significant difference observed after 24 hours. However, after 72 hours upon treatment there is significant decrease on cellular proliferation. Results represent three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test. Where  $P^{****}<0.0001$ ,  $P^{*}<0.01$ .



**Figure 4.8: LBH inhibitor phenocopies PAK6 knockdown cell morphology.** MDA-MB-231 cells were seeded on glass coverslips and treated with 5nM LBH or DMSO as a control for 24 and 48 hours. Quantified results represent three independent experiments. Bars are the standard error of the mean. Statistical significance was calculated using Student's *t*-test. Where  $P^{****} < 0.0001$ . Scale bar = 10  $\mu$ m.

#### **4.2.6. Active PAK6 can revert the LBH phenotype**

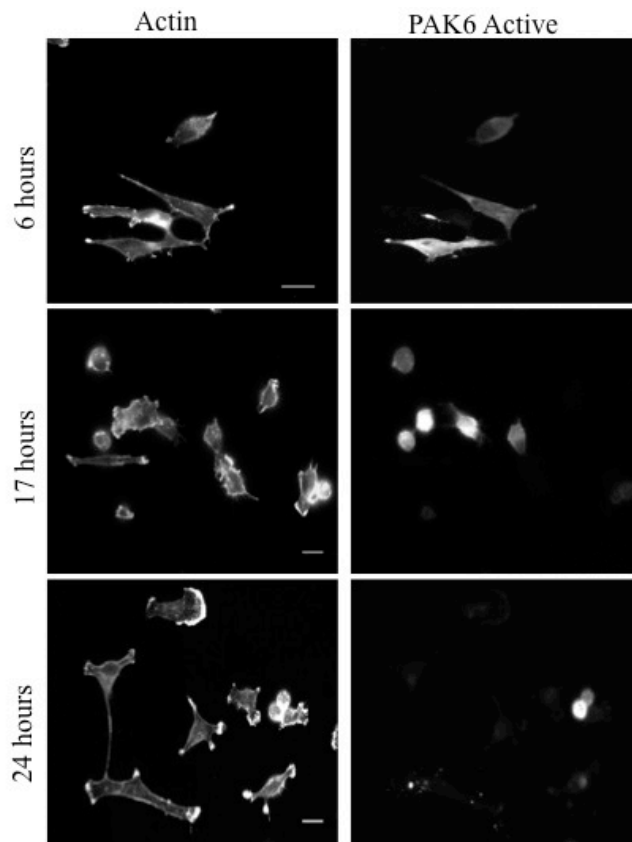
In these studies, the reduction of PAK6 expression either by siRNA or LBH treatment induced cell elongation. In contrast, the expression of active PAK6 (S531N) induced cell rounding. It was therefore reasoned that the expression of active PAK6 (S531N) might be able to rescue the morphological response to LBH. MDA-MB-231 cells were transfected with active PAK6 (S531N), wild type PAK6 and GFP alone for 24 hours to allow protein expression to occur and then treated with LBH inhibitor for 24 hours. Subsequently, the cells were fixed and stained for F-actin. The morphology of cells in all conditions was then analysed. LBH was able to induce cell elongation in both cells expressing GFP alone and PAK6. However, where cells were expressing active PAK6 (S531N), LBH treatment did not induce a significant level of cell elongation (Figure 4.9). Thus, active PAK6 (S531N) can revert the LBH phenotype.



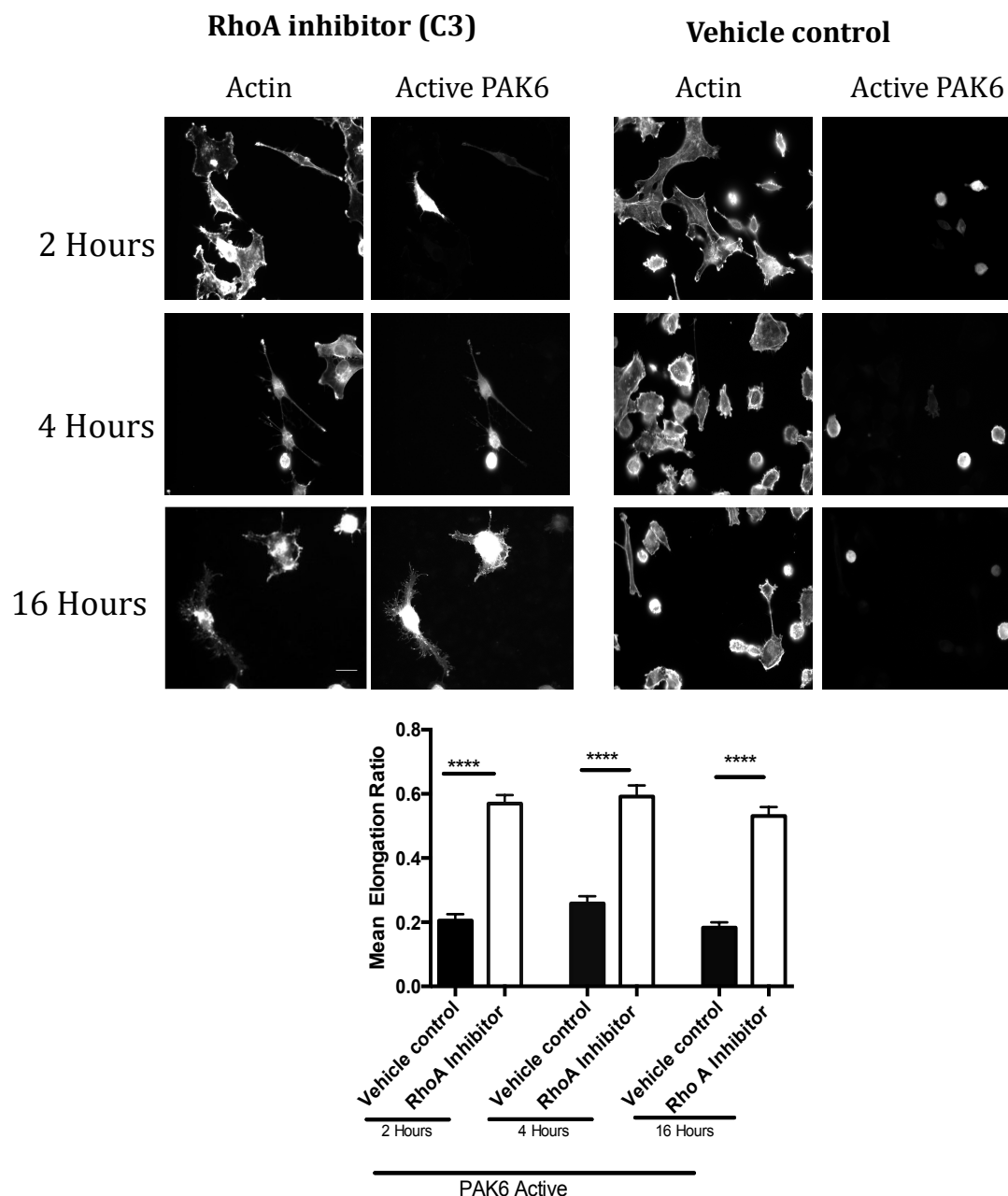
**Figure 4.9: Overexpress active PAK6 can revert the effect of LBH inhibitor.** MDA-MB-231 cells were seeded on coverslips and transfected with GFP as control, PAK6 WT, active PAK6 S531N and untransfected (UT) for 24 hours and then treated with LBH (5nM). Quantified data represents three independent experiments. Statistical significance was calculated using ANOVA *Tukey test*. Where  $P^{****} < 0.0001$ , n.s.=not significant. Scale bar=10 $\mu$ m.

#### **4.2.7. RhoA and ROCK but not Blebbistatin inhibitors prevent active PAK6 induced cell rounding**

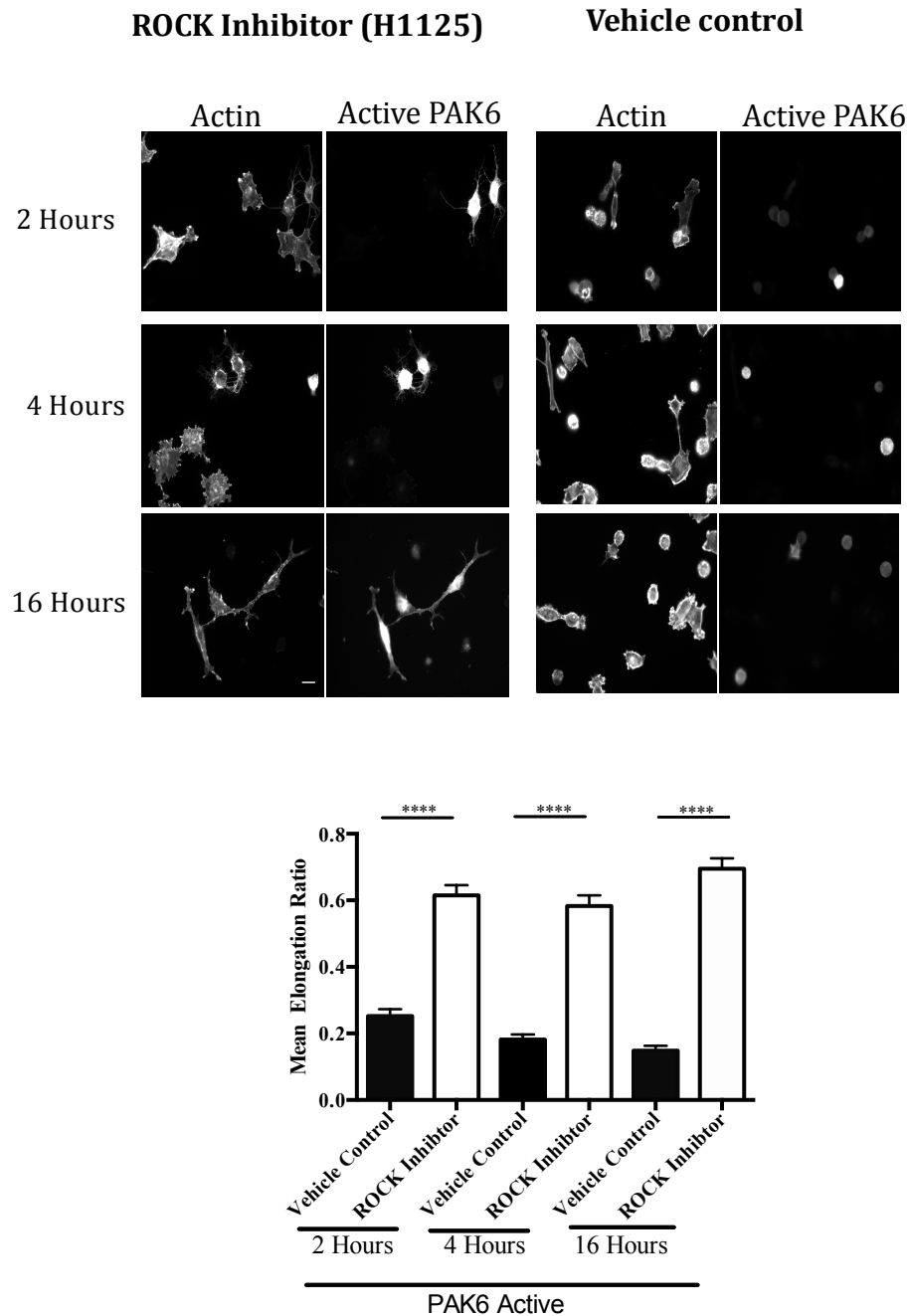
It is known that group I PAKs regulate myosin II contractility (Bokoch, 2003) and that PAK1 acts to coordinate the interplay between F-actin, myosin II and focal adhesion dynamics for efficient cell migration (Delorme-Walker et al., 2011). Here, the overexpression of PAK6 induces cell rounding (Figure 3.18), and therefore, PAK6 could be mediating cell contractility. Signalling via the Rho-ROCK pathway has been shown to be a key determinant of actomyosin contractility and is associated with rounded-amoeboid cell (Wilkinson et al., 2005b, Wolf et al., 2003). To explore further whether there is a mechanistic link between PAK6 and the Rho-ROCK pathway, a number of specific inhibitors were exploited. RhoA signalling was inhibited using the Rho specific inhibitor (C3), ROCK was inhibited using H1152 and Myosin II was inhibited by blebbistatin. It was decided to express active PAK6 in cells and then treat them with specific inhibitors to test for the ability of active PAK6 to induce cell rounding. Prior to the application of the inhibitors, the time point of cell rounding was determined. MDA-MB-231 cells were transfected with active PAK6 for 6, 17 or 24 hours. Studies suggest that cells were expressing active PAK6 6 hours following transfection but that cell rounding occurred between 6 and 17 hours post transfection (Figure 4.10). Thus, MDA-MB-231 cells were transfected with active PAK6, incubated for 6 hours and then treated with inhibitors or vehicle control. The results show that whilst treatment with vehicle control did not prevent active PAK6 induced rounding, treatment with C3 was able to prevent active PAK6 induced rounding and the cells remained significantly more elongated than vehicle control (Figure 4.11). Consistent with a Rho:ROCK pathway, the inhibition of active PAK6 expressing cells with H1152 also prevented cell rounding, and treated cells were again significantly more elongated than vehicle control (Figure 4.12). Interestingly, treatment with blebbistatin inhibitors whilst trending towards increased elongation at early time points was unable to significantly prevent active PAK6 induced cell rounding as seen with C3 and H1152 (Figure 4.13). Taken together, these data suggest that the active PAK6 induced rounded morphology is dependent on the Rho:ROCK pathway. However it is less clear how the cell rounding signal is transduced downstream of ROCK.



**Figure 4.10: PAK6 overexpression at different time points.** MDA-MB-231 cells were seeded onto glass coverslips and transfected with active GFP-PAK6 at different time points. Cells were then fixed after 24 hours and stain for actin. Scale bar = 10 $\mu$ m.

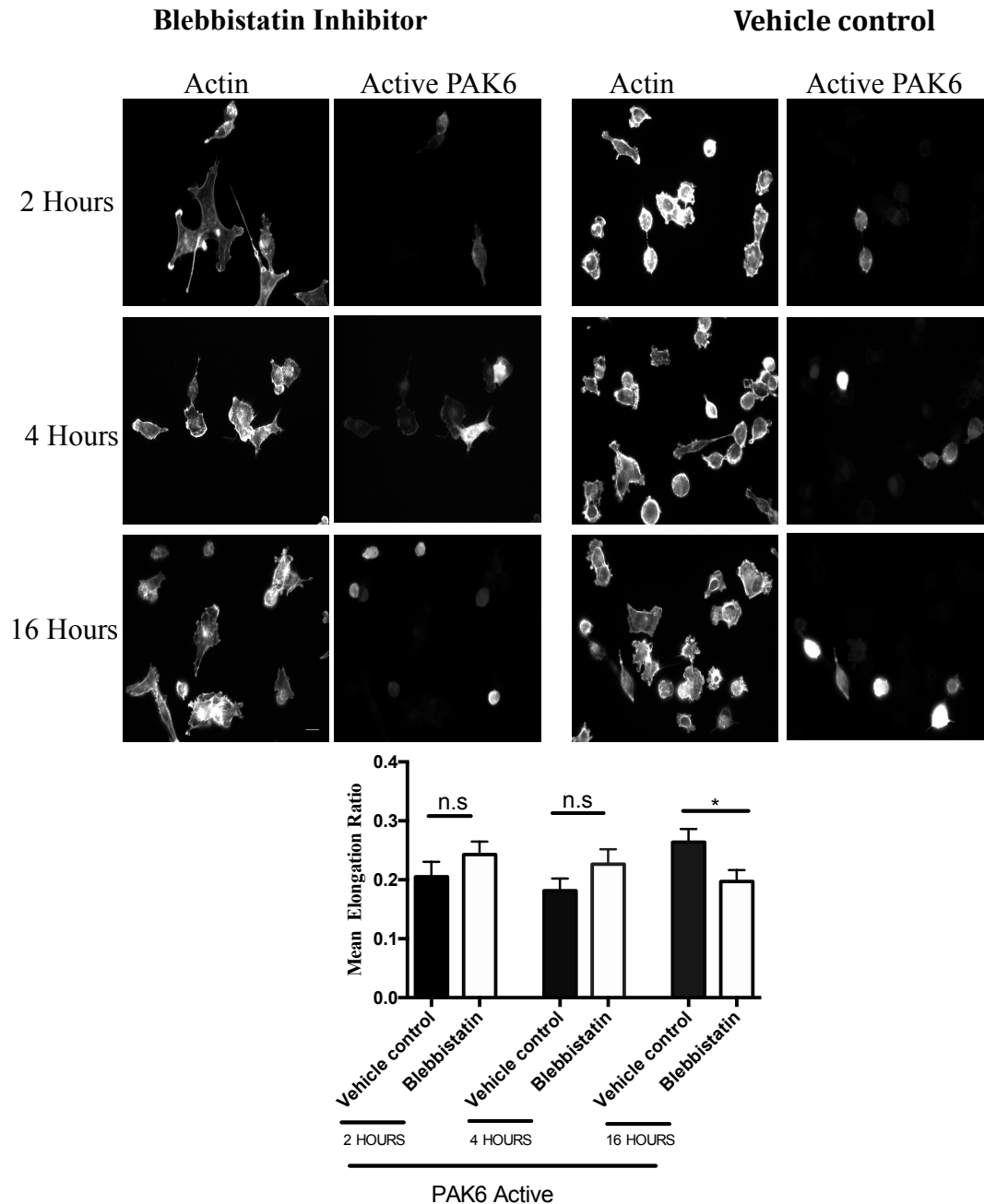


**Figure 4.11: RhoA inhibitor prevents active PAK6 induce cell rounding.** The effect of using 5 $\mu$ M RhoA inhibitor (C3) on cell morphology in MDA-MB-231 cells after 2,4 and 16 hours were investigated. MDA-MB-231 cells were seeded on coverslips; following day the cells transfected with Active PAK6 and treated with RhoA inhibitor at different time points. Quantification results represents three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test where  $P^{****}<0.0001$ .



**Figure 4.12: ROCK inhibitor prevents active PAK6 induce cell rounding.** The effect of using 5 $\mu$ M ROCK inhibitor (H1152) on cell morphology in MDA-MB-231 cells after 2,4 and 16 hours were investigated. MDA-MB-231 cells were seeded on coverslips; following day the cells transfected with Active PAK6 and treated with ROCK inhibitor for different time points. Quantification results represents three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test where  $P^{****}<0.0001$ .



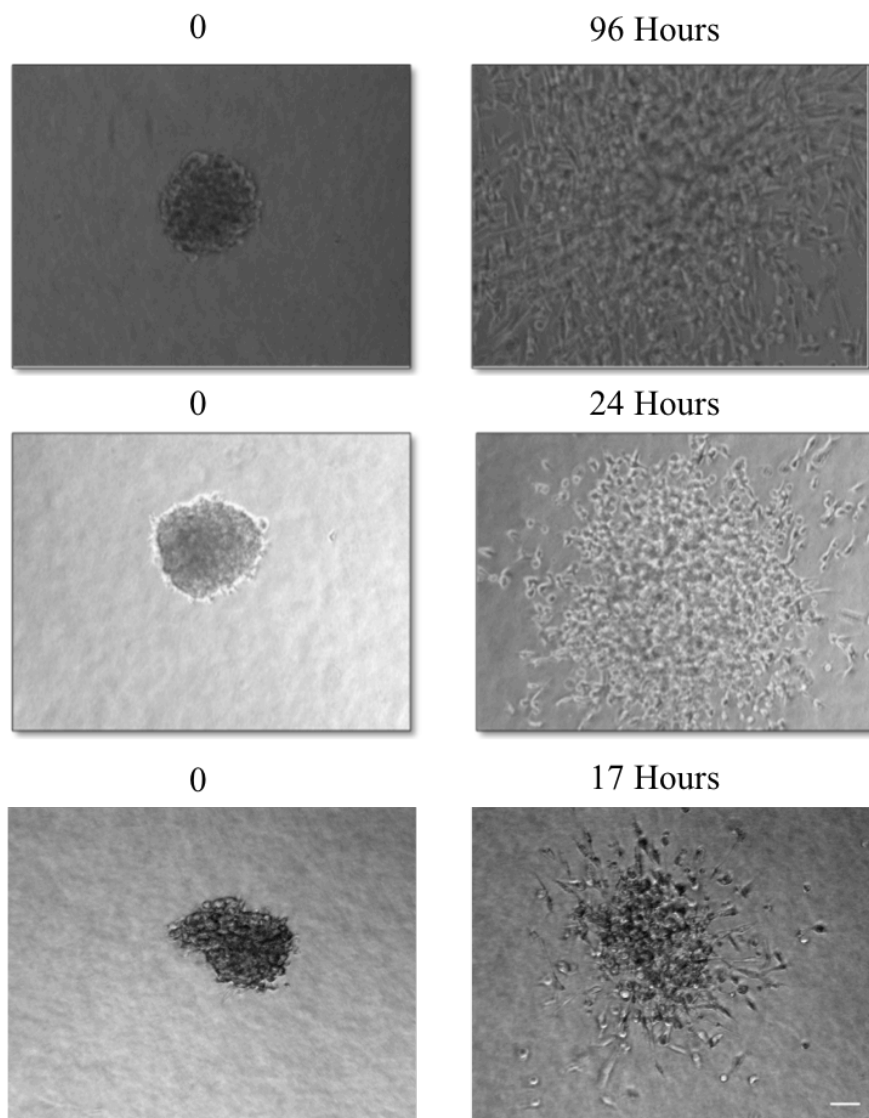


**Figure 4.13: Blebbistatin does not prevent active PAK6 induce cell rounding.** The effect of using 5 $\mu$ M Blebbistatin (MyosinII inhibitor) on cell morphology in MDA-MB-231 cells after 2,4 and 16 hours were investigated. MDA-MB-231 cells were seeded on coverslips; following day the cells transfected with Active PAK6 and treated with Blebbistatin and vehicle control and incubated at different time points. Quantification results represents three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test where  $P^* < 0.02$ , n.s|=not significant.

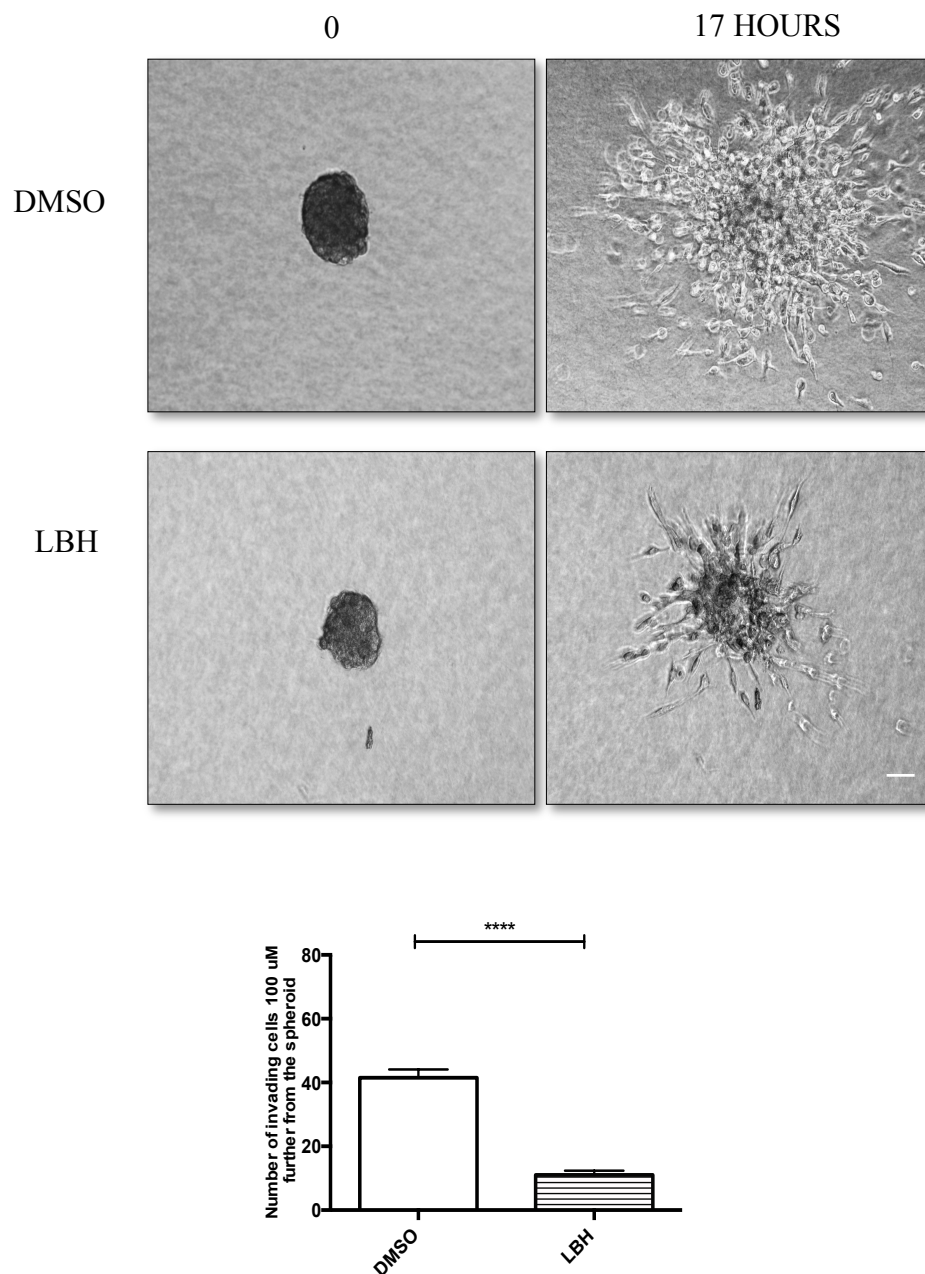
#### **4.2.8. Optimization the 3D spheroid invasion assay using MDA-MB-231 cells**

It is becoming increasingly apparent that the behaviour of cells in a 2D environment does not always reflect their 3D behaviour. Therefore, in parallel with 2D cell morphology studies, it was decided to employ 3D spheroid invasion assays. It was originally anticipated that this assay could be used to assess the effect of PAK6 knockdown on invasive migration. Prior to the use of PAK6 depleted cells, the assay was optimized for use with MDA-MB-231 cells, as this was a new technique introduced into the laboratory. In the spheroid assay, the spheroid tumour masses are created and encapsulated by a 3D matrix, such as collagen I, to represent the primary tumour mass and the surrounding tissue. Over time, the tumour cells invade away from the spheroid mass, and this invasion can be quantified.

To form the spheroid mass, cells were submerged in methylcellulose media for 3 days. The cell mass was then transferred into the collagen I matrix, where the level of invasion was quantified. Cell invasion was measured as the number of cells that had migrated further than 100  $\mu\text{m}$  from the spheroid edge. MDA-MB-231 cells were observed to invade relatively quickly compared to melanoma cells in our laboratory; whereas melanoma cells require 2-3 days embedding to deliver a quantifiable invasion, MDA-MB-231 cell invasion was visible 17 hours post embedding, as illustrated in Figure 4.14. Thus, the end point was modified for MDA-MB-231 cells. Unfortunately, it was not possible to test PAK6 depleted cells in the spheroid assay as the time frame for PAK6 knockdown was limited to 24 hours (Figure 4.1) and did not facilitate a long-term assay. However, it was decided to test the impact of LBH inhibitor treatment, as there was a slightly longer window of PAK6 depletion. MDA-MB-231 cells were treated with the LBH inhibitor and allowed to form a spheroid and the cells were then tested for invasion into the collagen matrix. Whilst DMSO treated control cells were able to successfully invade the collagen, and more than 40 single cells on average could be detected beyond 100 $\mu\text{m}$  from the spheroid center, LBH treatment had a significant inhibitory effect on the ability of these cells to invade (Figure 4.15).



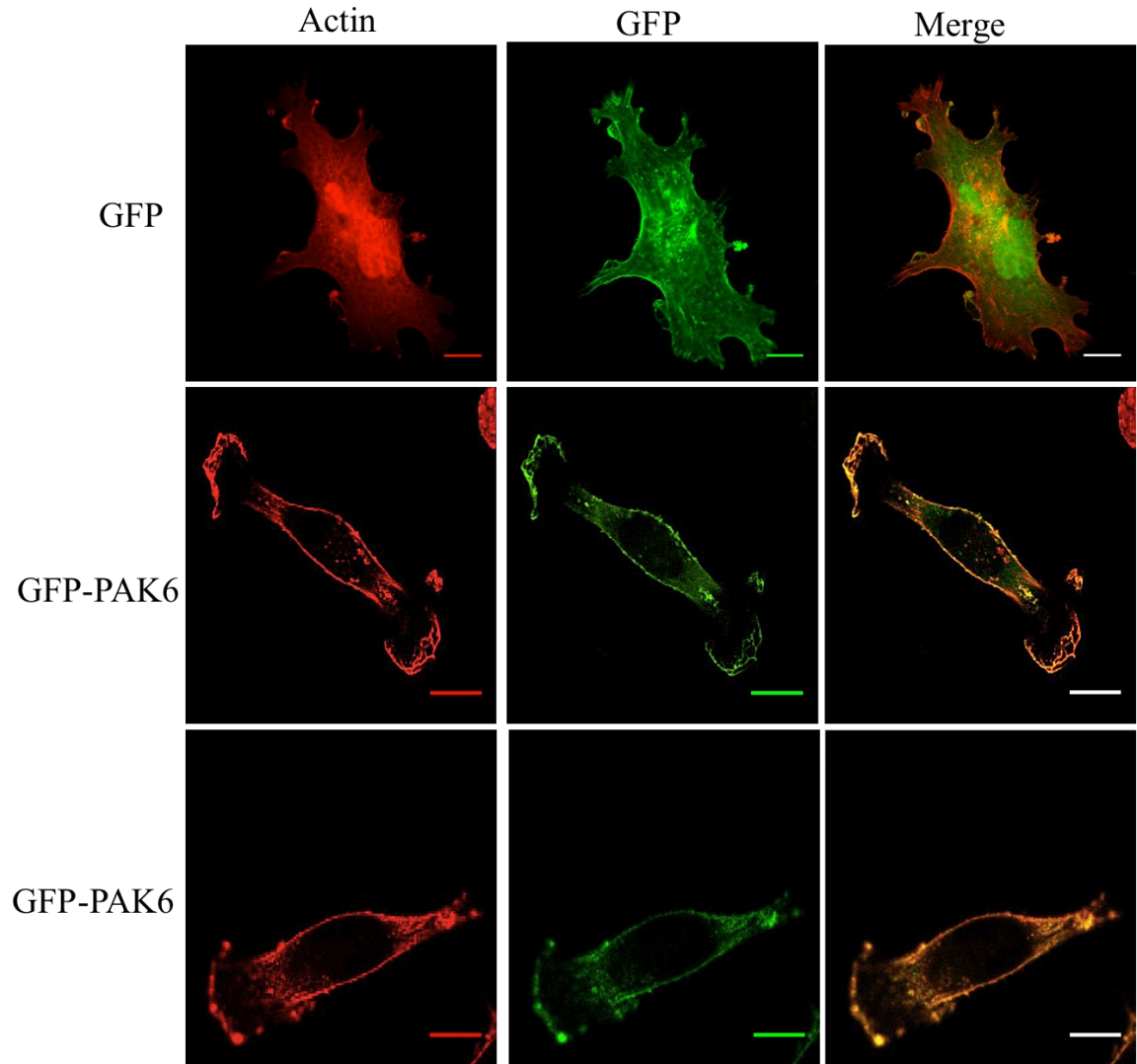
**Figure 4.14: Optimization of a 3D spheroid invasion assay using MDA-MB-231 cells.** Representative phase contrast images of the 3D spheroid invasion assay are shown. Spheroids were produced in media containing methylcellulose for 3 days, then submerged in collagen type I matrix. Phase contrast images were taken at day 0 and 96, 24 and 17 hours. The cell invasion was measured after 96, 24 and 17 hours commencing when the spheroids were placed in collagen type I.



**Figure 4.15: 3D spheroid invasion assay.** Representative image at day 0 and 17 hours of the 3D invasion assay in MDA-MB-231 cells in which the cells treated with DMSO as control and LBH. Spheroids were produced in media containing methylcellulose for 3 days, then submerged in collagen type I matrix. Phase contrast images were taken at day 0 and 17 hours. The cell invasion was measured after 17 hours commencing when the spheroids were placed in collagen type I. Bars represent the mean  $\pm$  S.E.M. Statistical significance was calculated using Student's *t*-test where  $P^{****} < 0.0001$ . Scale bar 30 μm.

#### **4.2.9. PAK6 is localised at the cell periphery in MDA-MB-231 cells**

The work presented here has identified a putative role for PAK6 in cell rounding mediated by the Rho:ROCK signalling pathway. Such a role might suggest a peripheral or cell adhesion localisation. However, very little is known about subcellular localisation of PAK6. It has been speculated that PAK6 is localized at the cell periphery in androgen expressing cells and subsequently translocates to the nucleus upon receptor stimulation (Yang et al., 2001). In contrast, previous work in our laboratory demonstrated that in colony forming prostate cancer cells, PAK6 localized at cell-cell boundaries (Fram et al., 2014). The localisation of PAK6 in MDA-MB-231 cells has not been previously investigated. Ideally, localisation would be established using an antibody to endogenous PAK6, however, no such antibody is available for Immunofluorescence. Thus, GFP-tagged PAK6 was utilized. GFP-PAK6 was transiently expressed in MDA-MB-231 cells, and the subcellular localisation observed by confocal microscopy. The F-actin staining revealed that GFP localizes to the cytoplasm and GFP-PAK6 localizes predominantly in the cytoplasm, however it was also possible to detect GFP-PAK6 at the periphery of the cells (Figure 4.16).



**Figure 4.16: Localisation of exogenous PAK6 in MDA-MB-231 cells.** MDA-MB-231 cells were transfected with GFP alone as a control and GFP-PAK6 wild type. After 24 hours cells were fixed and stained with TRITC-phalloidin to detect F-actin. Confocal images were taken. Scale bar 10 $\mu$ m. Image represents three independent experiments.

### 4.3. Discussion

In this chapter, the potential role of the PAK family member PAK6 on cell morphology has been further investigated. Complementary to Chapter 3, it has now been demonstrated that cells induce the opposing morphological change when PAK6 expression is depleted. Interestingly, the reduction of PAK6 expression using LBH inhibitor treatment phenocopies the morphology of PAK6 knockdown. Moreover, the expression of active PAK6 can prevent the LBH inhibitor induced cell morphology changes, whilst inhibition of the RhoA:ROCK pathway prevented active PAK6 induced morphologies. These results suggest that PAK6 may play an important role in the regulation of cell contractility and the cytoskeleton.

It is known that PAK6 is up regulated in various cancer cell lines and tissue samples (Chen et al., 2014, Liu et al., 2014, Kaur et al., 2008b). PAK6 depletion is known to inhibit cell growth, invasion and the ability to disassemble junctions in response to HGF in prostate cancer cells (Goc et al., 2013, Fram et al., 2014). However, there is no study addressing the effect of PAK6 knockdown in breast cancer cells. The level of PAK6 in MDA-MB-231 cells was significantly reduced when compared to PAK6 levels in control siRNA and untransfected (mock) samples. No significant difference in PAK6 expression levels were detected between mock and control siRNA in MDA-MB-231 cells. However, whilst PAK6 knockdown was achieved, this was a challenging process that required a series of experiments, using a range of siRNAs from different companies in order to obtain optimal knockdown efficiencies. PAK6 levels were significantly decreased after 24 hours siRNA treatment but started to recover after 48 and 72 hours of treatment. There are a number of reasons that could account for this relatively short period of PAK6 depletion. PAK6 was classed as a ‘difficult to silence’ gene in a siRNA screen where the central region of PAK6 mRNA was found to be highly difficult to target using siRNA knockdown (Bergauer et al., 2009). Conversely, it has been reported that PAK6 has anti apoptotic functions in prostate cancer cells (Zhang et al., 2010), perhaps MDA-MB-231 cells with a high level of PAK6 depletion are no longer viable.

In hepatocellular carcinoma (HCC) samples, PAK6 was overexpressed compared with non-cancerous liver tissue, and PAK6 was positively correlated with proliferation and poor prognosis (Chen et al., 2014). Moreover, in colon cancer PAK6 promotes tumour progression and chemoresistance, both *in vivo* and *in vitro* (Chen et al., 2015). However, a difference in cell proliferation rate was not observed in the PAK6 siRNA treated cells (Figure 4.3). Since a stable PAK6 knockdown cell line could not be generated there could be a long-term cell viability issue or alternatively, the case that PAK6 protein turnover occurs very quickly.

Despite the technical difficulties of working with PAK6 siRNA treated cells it was possible to conduct cell morphology assays by keeping to the small reduced expression window. The results show that in direct contrast to the overexpression of the active form of PAK6 (Figure 3.19), depletion of PAK6 significantly induces cell elongation in MDA-MB-231 cells (Figure 4.5). Interestingly, cells became much more elongated and larger in area following depletion of PAK6. The same phenotype was also observed in cells coated on collagen or fibronectin. This is confirming that the morphological changes observed are not dependent on matrix composition. It is known that cell spreading can be modulated by RhoGTPases and integrin-mediated signalling pathways (Herrera, 1998, Huttenlocher and Horwitz, 2011). RhoGTPases are necessary for the reorganization of the actin cytoskeleton during cell movement (Ridley et al., 1995), and integrins play an essential role in mediating the attachment of cells to the ECM (Herrera, 1998, Huttenlocher and Horwitz, 2011).

It is well established that the Rho family GTPases Rho, Rac and Cdc42 organise cell adhesion, turnover and cell migration (Ridley et al., 2003). High levels of Rho-ROCK signalling in tumour cells drive high levels of actomyosin contractility and are associated with the movement of individual cells in a rounded phenotype whereas lower levels of contractility are associated with Rac-dependent elongated movement. Having looked at the effect of PAK6 knockdown on cell morphology, the involvement of PAK6 in the Rho-ROCK pathway was investigated. The results show that cells expressing active PAK6 remain elongated in the presence of ROCK (H1152) and RhoA (C3) inhibitors but not blebbistatin. These data suggest that PAK6 could be involved in RhoA signalling pathways. Since PAK6 binds to Cdc42, but not RhoA (Schantz et al., 2004), these data raise the question as to whether the relationship between PAK6 and RhoA is direct or is mediated via other



mechanisms or other proteins. Interestingly the silencing of RhoA expression in MDA-MB-231 cells also induced cell elongation similar to PAK6 (Vega et al., 2012). It might be speculated that overexpression PAK6 activates a RhoA pathway, which leads to increased MLC2 phosphorylation, whereas the knockdown of PAK6 results in reduced activation of RhoA and phosphorylation of MLC2. However, the results from blebbistatin inhibition suggest that PAK6 induced cell rounding does not require the activity of Myosin II. PAK6 may be involved in other pathways downstream of ROCK. It is known that ROCK phosphorylates and activates LIMK, which in turn phosphorylates cofilin (Riento and Ridley, 2003).

It is possible that the cell rounding phenotype observed with PAK6 could also be the result of a loss of cell adhesion, as has been previously reported for PAK4 (Wells et al., 2010a). Perhaps, PAK6 links Cdc42 activation to integrin signalling. Whether PAK6 is involved in integrin signalling remains to be determined. However, it should be noted that the phenotype observed following the PAK6 depletion is not the same as the PAK4 depletion phenotype in MDA-MB-231 cells, suggesting a differential mechanistic pathway (Dart et al., 2015). It is reported that the rounded amoeboid cell movement is less dependent on integrin signalling than the mesenchymal type cell movement, and this might be relevant to the PAK6 induced phenotype (Wolf et al., 2003).

While the work presented here was being carried out, a new study was published demonstrating that the pan-deacetylase inhibitor Panobinostat (LBH) could suppress the expression of PAK6 in hepatocellular carcinoma cells (Henrici et al., 2013). Therefore, the effect of the LBH inhibitor on PAK6 expression and cell morphology was investigated. Consistent with PAK6 knockdown results, the inhibition of PAK6 expression leads to significantly elongated cells following LBH inhibitor treatment (Figure 4.8). In addition, active PAK6 overexpression can invert the LBH inhibitor cell phenotype. However, it should be considered that this inhibitor may not be specific for PAK6 and it is not really known how PAK6 expression can be down regulated by LBH.

Down regulation of PAK6 in the presence of the inhibitor could be the direct result of histone acetylation. Alternatively, hyperacetylation could result in the transcription of regulatory factors that negatively regulates gene transcription. It has been reported that in some prostate cancer cells the PAK6 gene is hypermethylated (Wen et al., 2009), which would ordinarily lead to down regulation of PAK6 expression. Nevertheless, the combined LBH and knockdown data strongly validate the cell elongation phenotype.

In addition to cell morphology studies, the LBH inhibitor was also utilized in 3D spheroid assays, as PAK6 knockdown cells could not be used. LBH treatment (including the depletion of PAK6 expression) leads to a significant decrease in cell invasion. This is an interesting observation that requires more investigation. Consistent with this observation, it has previously been shown that PAK6 knockdown in prostate cancer cells had a significant reduction in invasion when compared to control cells (Wen et al., 2009). Furthermore, knockdown of PAK6 in PC3 prostate cancer cells results in reduced expression of MMP9 (Goc et al., 2013). Interestingly, recent studies suggest that histone deacetylase inhibitors (like LBH) could limit the expression of the matrix metalloproteinase MMP9 (Bolden et al., 2006). Thus, it cannot be ruled out that the inhibition of invasion delivered by LBH could be an indirect effect on the MMP, which may or may not be mediated by PAK6. It is interesting to note that in a panel of twelve melanoma cell lines, a higher secretion of several MMPs was observed including MMP9, MMP2 and MMP13, which were associated with rounded cell morphology when compared with elongated cells. MMP9 acted as a signalling intermediate to activate MLC2 phosphorylation and resulted in efficient rounded 3D cell migration (Orgaz et al., 2014). Thus, perhaps there is an intrinsic link between cell rounding, PAK6, ROCK, MMP expression and invasion.

Results presented here suggest that PAK6 can act as a cytoskeletal regulator and may be active in the cytoplasm and/or near to the plasma membrane. However, very little is known about PAK6 localisation in breast cancer cells. In non-colony forming fibroblastic CV-1 cells, the full-length PAK6 was found to be localized predominantly in the cytoplasm. PAK6 can also translocate to the nucleus with AR stimulation (Yang et al., 2001) in prostate cancer cells. PAK6 is also found localized at the plasma membrane in HeLa cells (Lee et al., 2002),

although in an independent study, PAK6 frequently localized to punctate formations in the cytoplasm of HeLa cells and NCI-H1299 lung cancer cells (Shepelev and Korobko, 2012). Our laboratory has previously reported that in colony forming prostate cancer cells, PAK6 localizes to cell-cell junctions (Fram et al., 2014). Since a PAK6 antibody that could be used for immunofluorescence experiments could not be generated, the localisation of exogenous tagged PAK6 was investigated. The localisation of PAK6 was in agreement with previous studies being mainly cytoplasmic, however, a distinct localisation at the cell periphery could be detected. This peripheral localisation might represent the active pool of PAK6 however without specific probes to localise active PAK6, this remains to be determined. It might be speculated that the induction of cell rounding could be delivered by modifications to the peripheral actin cytoskeleton.

In summary, it has been determined that there is an important specific role for PAK6 in mediating cell morphological changes using overexpression or knockdown strategies. Indeed, the work presented here suggests that PAK6 may mediate cell cytoskeleton reorganization. These PAK6 functions may be the result of interaction with other binding proteins. Further studies on the links between PAK6 and the RhoA signalling pathway are also required.

#### **4.4. Future works**

One major challenge with the work performed in this chapter was the time point of the knockdown and the transient nature of that knockdown. It would be interesting to explore the PAK6 lifetime cells through pulse-chase experiments. The knockdown PAK6 cells did show a clear phenotype; however, ideally, it would be better to produce a stable knockdown cell line, which was the original experimental plan to work with 3D and *in vivo* experiments. Given that attempts with shRNA did not yield successful long-term knockdown, it might be advantageous to exploit a newer technology, CRISPR. Although it cannot be assumed that a complete loss of PAK6 might be deleterious to the cells. CRISPR would also permit the re-expression of tagged PAK6 at endogenous levels (knock-in), which would enable the more accurate localisation of PAK6 in the breast cancer cells.

## **Chapter 5:**

### **Identification PAK6 binding partners**

## **5. Chapter 5: Identification PAK6 binding partners**

### **5.1. Introduction**

The work in Chapter 3 and 4 suggests that PAK6 interacts with actin cytoskeletal signalling pathways, which are recognised to be regulated by Rho family GTPases, such as Rho, Rac and Cdc42. It is widely established that Rac and Cdc42 bind to PAK family proteins. Indeed, for Group I PAKs, binding stimulates their kinase activity (Eswaran et al., 2008). The Group II PAKs are thought to preferentially bind to Cdc42, however, whether this Cdc42 interaction increases kinase activity is controversial (Schantz et al., 2004). Thus, the differential preference for Cdc42 and the controversy over activation suggest that the two groups of PAKs are regulated in fundamentally different ways (Abo et al., 1998, Arpino et al., 2004, Cotteret and Chernoff, 2006, Ha et al., 2012). Whilst binding to Rac and Cdc42 has been extensively explored, other RhoGTPases have been less studied, including the atypical RhoGTPases, such as RhoU and RhoV. These GTPases are of particular interest, as RhoU is reported to bind to PAK1 (Fort et al., 2011b), PAK4 (Dart et al., 2015) and PAK6 (Shepelev et al., 2011). The PAK6 interaction with RhoV is only reported in one study of human embryonic kidney (HEK) 293 cells, whilst in the same study RhoU was also reported to bind to PAK6 (Shepelev and Korobko, 2012). Moreover, it is reported that the overexpression of active RhoU can induce cell rounding in NIH3T3 cells (Chuang et al., 2007). In contrast, the overexpression of RhoV leads to lamellipodia formation, focal adhesion assembly and oncogenic transformation in NIH3T3 cells (Aronheim et al., 1998).

As downstream effectors of RhoGTPases Cdc42 and Rac1, PAK signalling is known to mediate multiple pathways, including cell movement. In the majority of cases, PAK drives these responses via substrate phosphorylation, and a number of PAK substrates have been identified (Chapter1, Table 1.1). In the context of migration it is reported that PAKs are linked to the regulation of actin cytoskeletal reorganization via phosphorylation of LIMK, GEF-H1 and MARK2 (Van Troys et al., 2008, Callow et al., 2005a, Zenke et al., 2004b, Matenia et al., 2005).

An interesting downstream target of PAK1 is the actin-binding protein FilaminA. FilaminA has been implicated in the regulation of cell migration, integrin-mediated adhesion, cell–cell adhesion and signal transduction (Chen et al., 2009, Zhou et al., 2010). The relationship between FilaminA and PAK is complex, with both upstream and downstream interactions reported, both from a single study (Vadlamudi et al., 2002b). On the one hand, FilaminA binds PAK1 in the CRIB domain, releasing autoinhibition and thereby stimulating PAK1 activity, on the other hand, PAK1 can phosphorylate FilaminA at serine 2152, which appears to drive the formation of dorsal membrane ruffles (Vadlamudi et al., 2002b). In contrast to the other PAK members, there is much less known about the interaction between PAK6 and the proteins controlling cytoskeletal dynamics. PAK6 can interact with IQGAP1, but this interaction was detected in the junction of colony forming cells (Fram et al., 2014, Kaur et al., 2008b).

The work presented in this chapter sought to further explore the interaction between PAK6 and the wider RhoGTPase family as well as to identify novel PAK6 binding partners that could reveal mechanistic insight into how PAK6 is interacting with cytoskeletal dynamics.

## **5.2. Results:**

### **5.2.1. PAK6 interacts with RhoV, RhoU and RhoD**

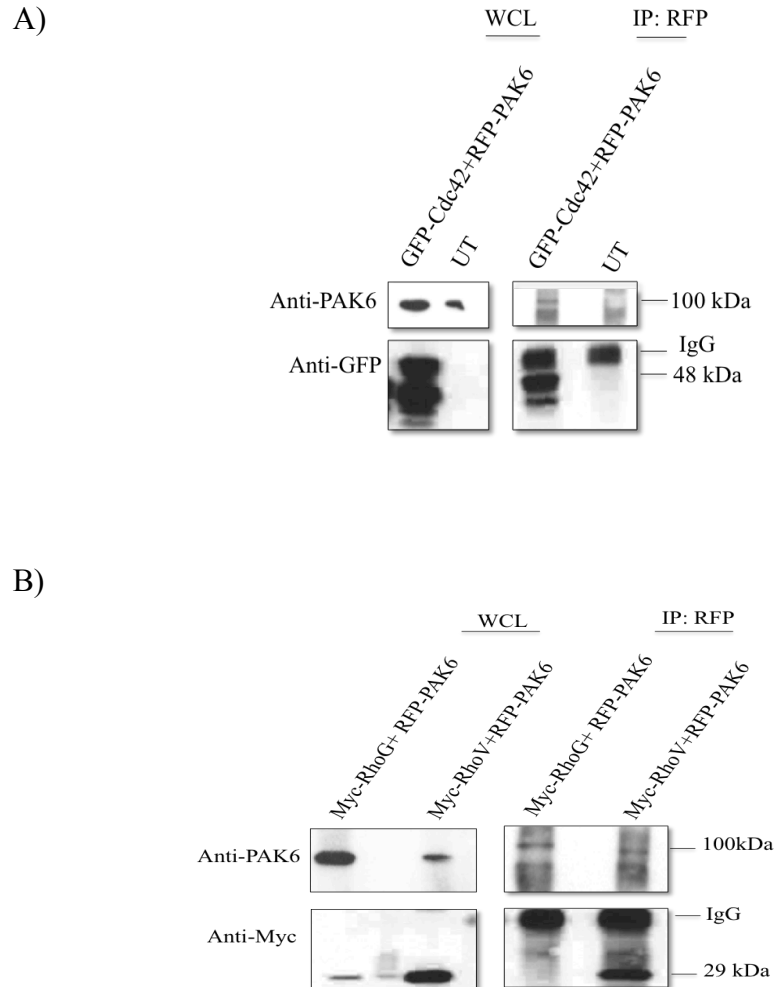
To explore the interaction between PAK6 and Rho Family GTPases in more detail, interaction studies were conducted in HEK293 cells. This system was adopted, as it yields high levels of protein expression and facilitates the detection of protein:protein interaction by immunoprecipitation. Initially, cells were co-transfected with GFP-Cdc42 and RFP-PAK6 as a positive control, as this was tested against Myc-RhoV, which is already published to interact, and Myc-RhoG, which as a close relative of Rac would be predicted not to bind PAK6. In this experiment, RFP-PAK6 was immunoprecipitated with anti-RFP antibodies, and the immune complexes were then analysed by western blotting for the expression of RFP-PAK6 (anti-PAK6) and RhoGTPases using anti-GFP (Figure 5.1 A) and anti-Myc antibodies (Figure 5.1 B). The results show as expected, Cdc42 bound to PAK6, confirming the validity of the assay (Figure 5.1 A). Moreover, in agreement with the previous study (Shepelev and Korobko, 2012), PAK6 interacted with RhoV (Figure 5.1 B), and as predicted did not interact with RhoG (Figure 5.1 B). These findings suggest that PAK6 might indeed be an effector of RhoV.

To further validate this result, an alternative strategy was employed using GFP-TRAP. Cells were transfected with GFP-PAK6 alone or co-transfected with GFP-PAK6 and Myc-RhoV. In addition, Myc-RhoU was also co-transfected with GFP-PAK6 to test this reported interaction (Shepelev and Korobko, 2012). The results confirm the interaction between PAK6 and RhoV or RhoU (Figure 5.2). However, despite an equal amount of overexpressed RhoV and RhoU in the cell lysates in the TRAP experiment, GFP-PAK6 appears able to complex with RhoV more efficiently than RhoU. This suggests that the interaction between RhoU and PAK6 may be weaker.

Having established PAK6 binding of Cdc42, RhoV and RhoU, an additional Rho family GTPase was tested, RhoD. RhoD has been linked to FilaminA activity (Gad et al., 2012) and is also thought to be related to Cdc42 at the cell response level (Ridley, 2006), playing a role in cell protrusion. Cells were co-transfected with GFP-RhoD and RFP-PAK6 and immunoprecipitated using an anti-RFP antibody. The complex was analysed by western

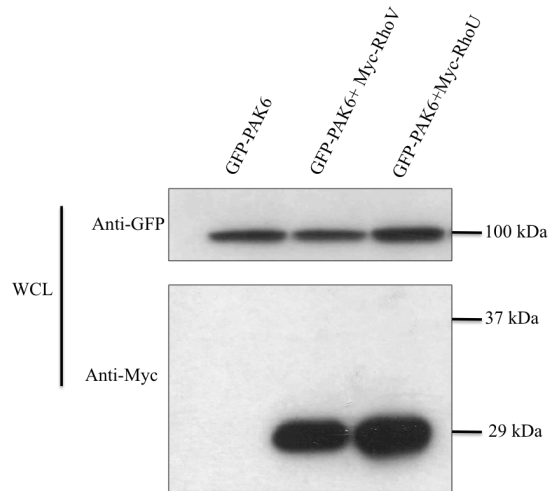


blotting using anti-PAK6 and anti-GFP antibodies (Figure 5.3), and GFP-RhoD was clearly detected in the PAK6 immunoprecipitation. To further validate this result, GFP-TRAP experiment was employed. Cells were co-transfected with GFP-RhoD and Myc-PAK6 or Myc-PAK6 alone. The results confirm the interaction between PAK6 and RhoD (Figure 5.4).

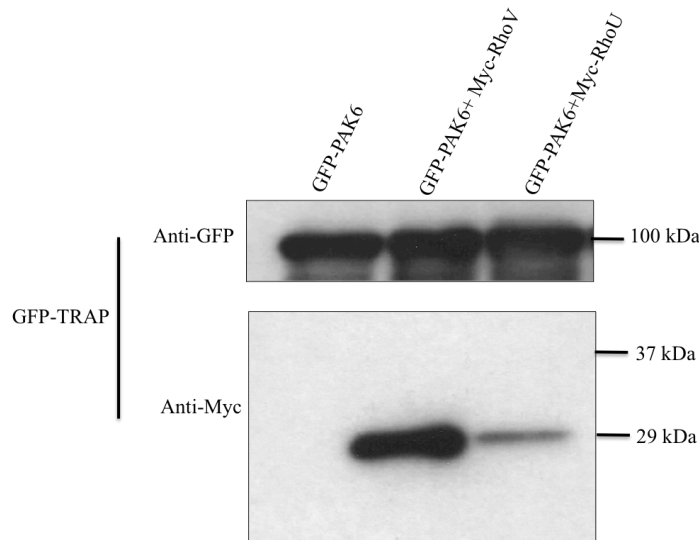


**Figure 5.1: Co-immunoprecipitation of PAK6 with Cdc42, RhoV and RhoG.** **A)** HEK293 cells were transfected with RFP-PAK6, and GFP-Cdc42 as indicated as positive control and untransfected as negative control. The cells were lysed and RFP-PAK6 was immunoprecipitated using an anti-RFP antibody (IP) from cell lysates. The samples were immunoblotted for overexpressed GFP-Cdc42 using an anti-GFP antibody, for RFP-PAK6 using an anti-PAK6. Whole cell lysates (WCL) were immunoblotted using anti-PAK6 and anti-GFP antibodies. **B)** The samples were immunoblotted for over- expressed c-Myc RhoV and c-Myc RhoG using an anti-c-Myc antibody, for RFP-PAK6 using an anti-PAK6. Whole cell lysates (WCL) were immunoblotted using anti-PAK6 and anti-c-Myc antibodies. The molecular weight for Cdc42 21kDa, RhoV 29 kDa and PAK6 75 kDa. The blots shown are representative of three independent experiments.

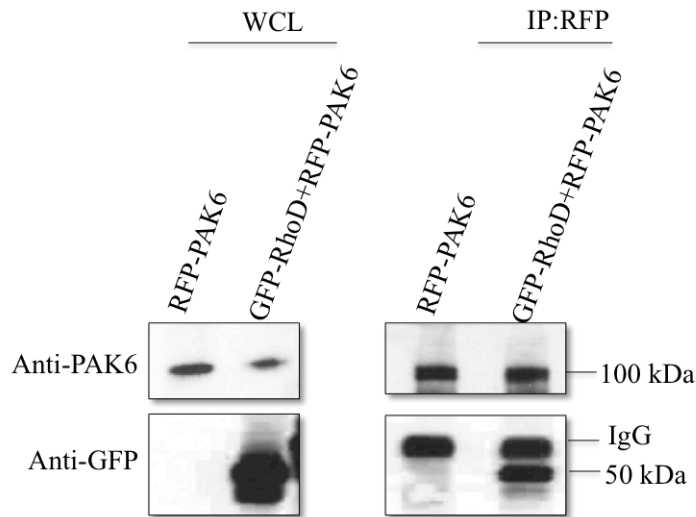
A)



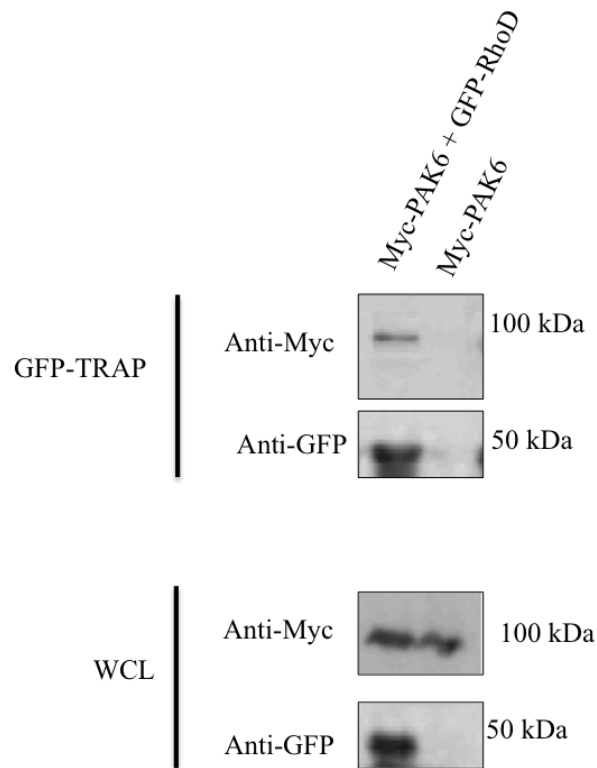
B)



**Figure 5.2: RhoV and RhoU binds to PAK6.** **A)** HEK293 cells were transfected with GFP-PAK6 alone, GFP-PAK6 and Myc-RhoU or GFP-PAK6 and RhoV. The cells were lysed and GFP-TRAP used. The samples were immunoblotted for over-expressed PAK6 using an anti-GFP antibody, for RhoU and RhoV using anti-Myc. **B)** Whole cell lysates (WCL) were immunoblotted using anti-GFP and anti-Myc antibodies. The molecular weight for PAK6 is 75 kDa, RhoU 28 kDa and RhoV 29 kDa. The blots shown are representative of three independent experiments.



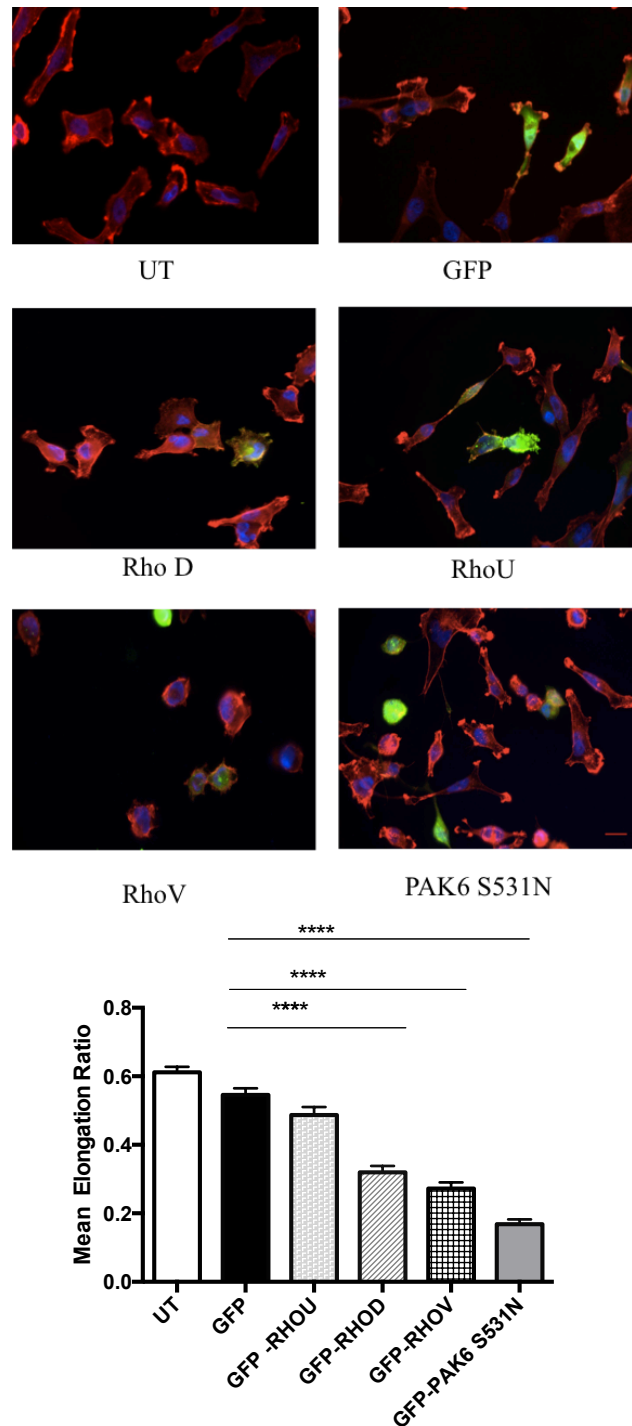
**Figure 5.3: Co-immunoprecipitation of PAK6 with RhoD.** HEK293 cells were transfected with RFP-PAK6 alone, or RFP-PAK6 with RhoD. The cells were lysed and RFP-PAK6 was immunoprecipitated using an anti-RFP antibody (IP) from cell lysates. The samples were immunoblotted for overexpressed RhoD using an anti-GFP antibody, for RFP-PAK6 using an anti-PAK6. Whole cell lysates (WCL) were immunoblotted using anti-PAK6 and anti-GFP antibodies. The molecular weight for RhoD is 23 kDa and PAK6 75 kDa. The blots shown are representative of three independent experiments.



**Figure 5.4: RhoD binds to PAK6.** HEK293 cells were transfected with Myc-PAK6 alone, Myc-PAK6 and GFP-RhoD. The cells were lysed and GFP-TRAP used. The samples were immunoblotted for over-expressed PAK6 using using an anti-Myc antibody, for RhoD using anti-GFP. Whole cell lysates (WCL) were immunoblotted using anti-GFP and anti-Myc antibodies. The blots shown are representative of three independent experiments.

### **5.2.2. MDA-MB-231 cell morphology is affected by RhoV and RhoD**

It was already established that the overexpression of active PAK6 could induce cell rounding (Chapter 3, Figure 3.19). PAK6 is not thought to be activated by Cdc42 (Schantz et al., 2004), but it may be that other RhoGTPases act to stimulate PAK6. Having established that PAK6 also interacts with RhoU, RhoV and RhoD, the impact of GTPase expression was tested to elucidate any commonality with activated PAK6. Overexpression of RhoU did not phenocopy active PAK6 overexpression; in contrast, overexpression of RhoV and RhoD induced a rounded phenotype when compared with GFP or untransfected control cells (Figure 5.5).



**Figure 5.5: RhoD and RhoV overexpression induces morphological changes in MDA-MB-231 cells.** MDA-MB-231 cells were transfected with GFP (control) or GFP-RhoU, GFP-RhoD, GFP-RhoV or active PAK6 S531N. After 24 hours, cells were fixed and stained with TRITC-phalloidin for F-actin. Shape analysis was performed on the cells using Image J to determine the elongation ratio. The quantification results over three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance where compared with GFP control cells was calculated using Student's *t*- test; \*\*\*\*,  $P < 0.0005$ . Bar = 10  $\mu$ m.

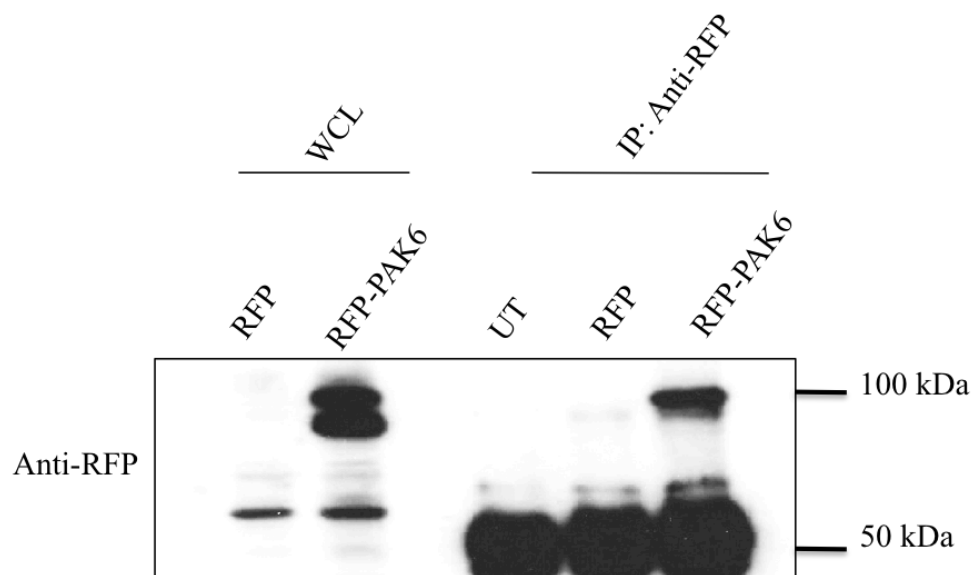
### **5.2.3. Identification of novel PAK6 binding partners by mass spectrometry**

In parallel to identifying RhoGTPase family members that can interact with PAK6, additional novel PAK6 binding partners were sought, with a specific aim is to identify breast cancer relevant interactions. To achieve this objective, PAK6 was isolated from breast cancer cells for interaction analysis. Initial work focused on developing good transfection and expression of PAK6 in selected breast cancer cells, and the testing whether PAK6 could be successfully isolated from lysates. RFP and RFP-PAK6 were transfected into MDA-MB-231 cells, immunoprecipitated using RFP antibody and PAK6 immunoblotted using anti-RFP antibody. The results show that PAK6 can be immunoprecipitated from MDA-MB-231 cells (Figure 5.6) with no band detected in untransfected and RFP control lane. The expression of PAK6 in the whole cell lysates consistently revealed a double band, which might be due to non-specific binding or degradation. The amount of PAK6 immunoprecipitated from cell lysates was not considered optimal for mass spectrometry analysis and the presence of a doublet was also troubling. Therefore it was decided to use GFP-TRAP beads as an alternative strategy as good yields had been achieved in HEK293 cells (Figure 5.3). Western blot analysis suggests that there was a good yield of GFP-PAK6 (single band) and very little background contamination. Moreover, unlike immunoprecipitation, the bound fraction of the GFP-TRAP does not contain contaminating polypeptide chains (antibodies). Thus GFP-TRAP was used for the mass spectrometry experiments.

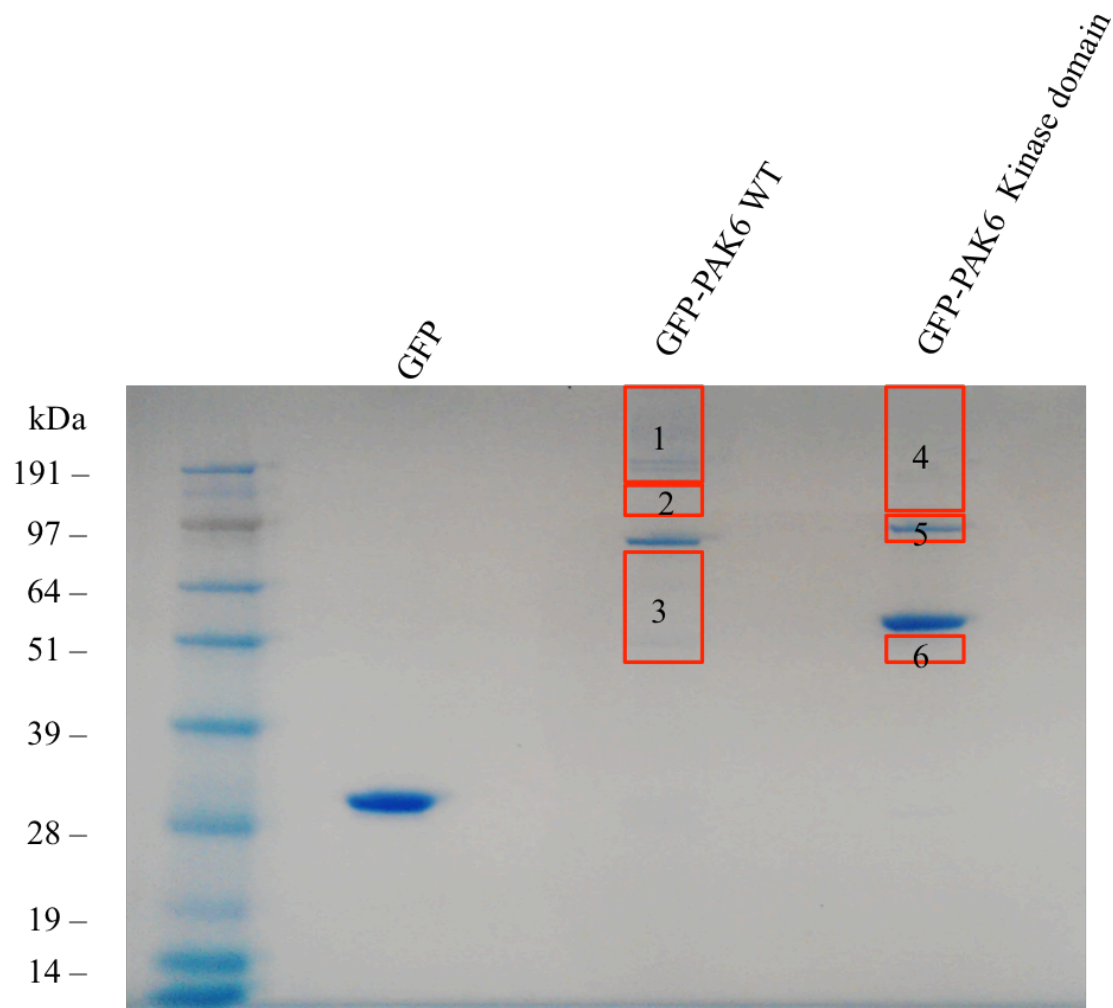
To identify novel proteins that interact with PAK6, MDA-MB-231 cells were transfected with wild type GFP-PAK6, GFP-PAK6 kinase domain or GFP alone to provide a control. This approach allowed detection of proteins that bind to the PAK6 kinase domain, which could represent a substrate or detection of proteins that bind outside the kinase domain, which could represent regulators. The isolated protein complexes were first resolved using SDS-PAGE gel and stained with Coomassie blue (Figure 5.6).



Protein bands were excised, digested with trypsin and proteins identified by Liquid chromatography–mass spectrometry (LC/MS). The mass spectrometry was performed by Malcolm Ward’s group based at King’s College London. The criteria applied were to exclude the PAK6 overexpression protein band and proteins at the level of RhoGTPases as this would skew the analysis towards known interactions. Analysis was restricted to the protein bands in the boxes as illustrated in (Figure 5.7)



**Figure 5.6: PAK6 immunoprecipitated from MDA-MB-231 cells.** MDA-MB-231 cells were transfected with RFP-PAK6 and RFP as control untransfected (UT). The cells were lysed and overexpressed PAK6 was immunoprecipitated using anti-RFP antibody (IP). IPs was immunoblotted using anti-RFP antibodies. The blot shown is representative of three independent experiments.



**Figure 5.7: Identification of novel PAK6 binding partners expressed in MDA-MB-231 cells by mass spectrometry.** MDA-MB-231 cells were transfected with GFP as control, GFP-PAK6 or GFP-PAK6 Kinase domain. Cells were lysed and add GFP-TRAP beads. The gel runs over three independent experiments.

#### 5.2.4. FilaminA, MyosinIIA and 14-3-3 proteins interact with PAK6

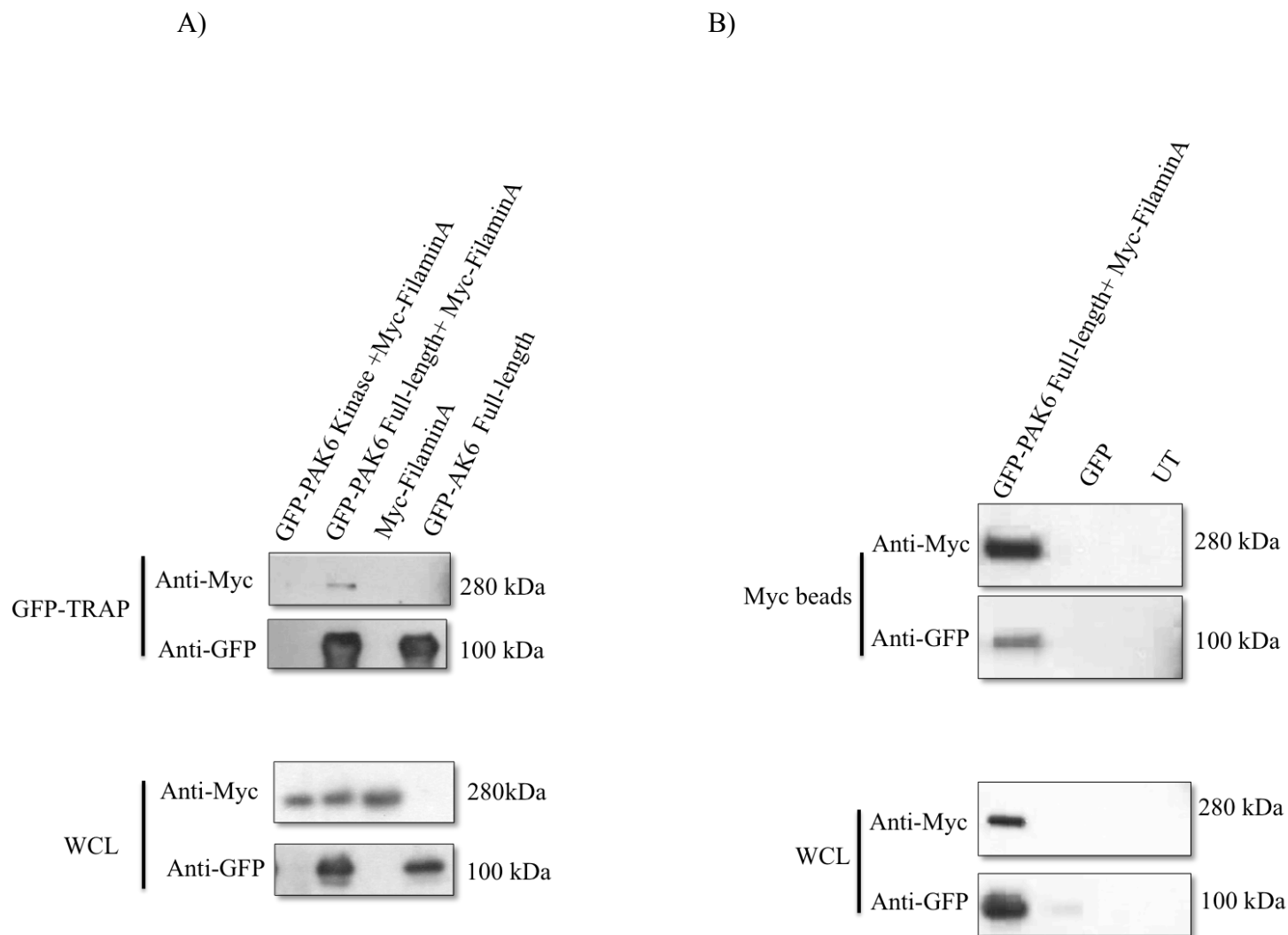
The results from mass spectrometry analysis indicate some novel proteins that bind to PAK6 (Table 5.1) but not to GFP alone. Interestingly, the experiments also revealed some proteins that bind to wild type full-length PAK6 but not to the PAK6 kinase domain and vice versa. FilaminA, Myosin IIA and 14-3-3 proteins were found to bind to the wild type PAK6. Others have shown recently shown that 14-3-3 can also bind to PAK6 in HEK293 cells (Tinti et al., 2014). Any putative interaction partner identified either through affinity purification or biochemical fractionation must be validated to confirm its physiological relevance. The interaction between PAK6 and Myosin IIA could not be confirmed. Given that the interaction between PAK6 and 14-3-3 had already been reported (Tinti et al., 2014), and that GEF-H1 has already been extensively characterised with respect to PAK (Callow et al., 2005a, Zenke et al., 2004b), it was decided to focus on the FilaminA and PAK6 interaction.

**Table 5.1:** The results from mass spectrometry.

	<b>GFP</b>	<b>GFP-PAK wild type (Full-length)</b>	<b>GFP-PAK6 Kinase domain alone</b>
<b>FilaminA</b>	NO	Yes	NO
<b>Myosin IIA</b>	NO	Yes	NO
<b>14-3-3 protein</b>	NO	Yes	NO
<b>GEF H1</b>	NO	NO	Yes
<b>Vimentin</b>	Yes	Yes	Yes

### **5.2.5. Full-length PAK6 interacts with FilaminA**

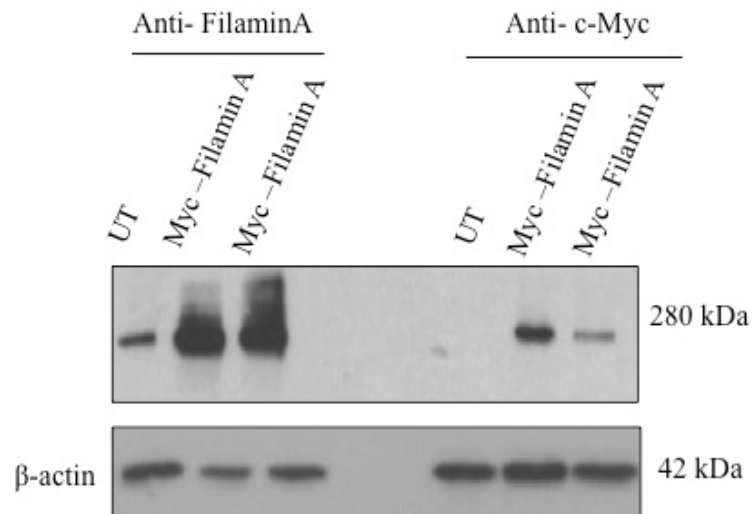
For initial validation of a specific interaction between PAK6 and FilaminA, HEK293 cells were used. FilaminA is a large (approximately 280 kDa) multi-domain scaffold protein that is known to have multiple binding partners (van der Flier and Sonnenberg, 2001). HEK293 cells were transfected with full-length GFP-PAK6 and Myc-FilaminA, GFP-PAK6 kinase domain and Myc-FilaminA, GFP-PAK6 full-length alone or Myc-FilaminA alone. Consistent with the mass spectrometry predictions FilaminA was isolated with full-length PAK6 but not the kinase domain (Figure 5.8 A). To confirm this result, an alternative protocol was used to test the specificity of this interaction. HEK293 cells were transfected with Myc-FilaminA and either full-length GFP-PAK6 or GFP alone. Myc beads were then added to pull down Myc-FilaminA and western blot analysis revealed that full-length PAK6 was also present in the complex (Figure 5.8 B).



**Figure 5.8: PAK6 interacts with FilaminA in HEK293 cells.** **A)** HEK293 cells were transfected with GFP-PAK6 full-length and Myc-FilaminA, GFP-PAK6 kinase domain and Myc-FilaminA, Myc FilaminA and GFP-PAK6 full-length. The cells were lysed and GFP-TRAP used and immunoblotted for FilaminA using anti-Myc and for PAK6 using anti GFP. Whole cell lysate was immunoblotted for FilaminA using anti-Myc and for PAK6 using anti GFP. The blots are representative of three independent experiments. **B)** HEK293 cells were transfected with GFP-PAK6 full-length and Myc-FilaminA and GFP alone. The cells were lysed and Myc beads used and immunoblotted for FilaminA using anti-Myc and PAK6 using GFP antibody representative of two independent experiments.

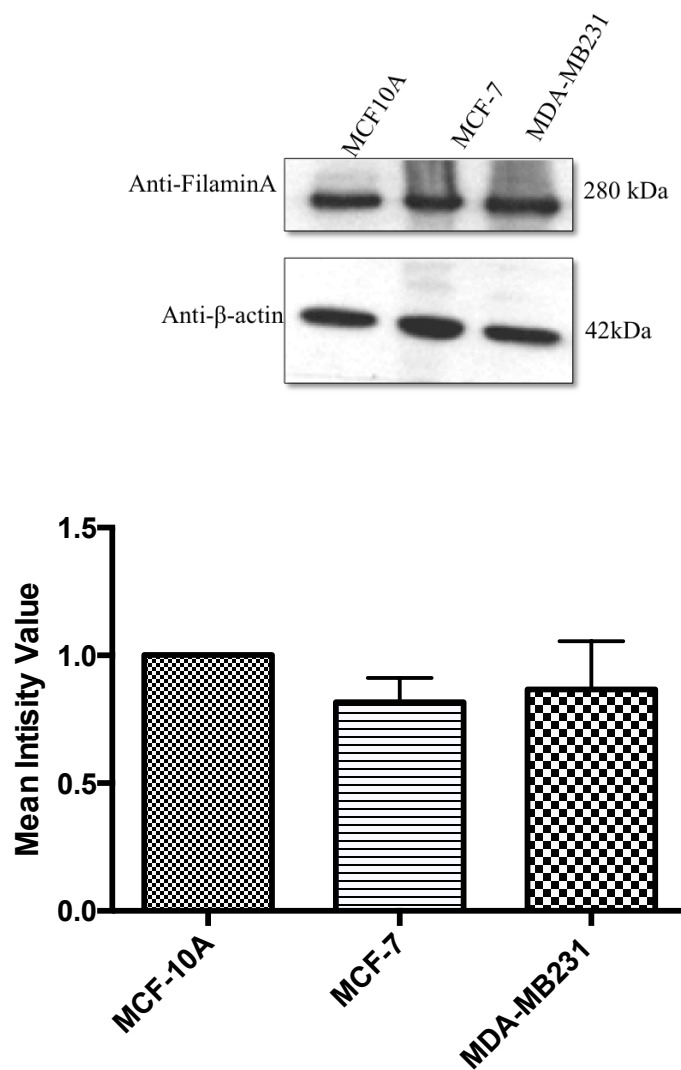
### **5.2.6. FilaminA is expressed endogenously in breast cancer cell lines**

Although PAK1 has been reported previously to interact with FilaminA (Vadlamudi et al., 2002b), this finding is a novel interaction for PAK6. An anti-FilaminA antibody was obtained from Gentex and detected band at the correct size for Myc-tagged FilaminA when overexpressed in MDA-MB-231 cells (Figure 5.9). Subsequently the antibody was tested for efficient detection of endogenous FilaminA in our chosen cells line (MDA-MB-231) and expression levels of FilaminA compared to normal non-tumorigenic MCF10A and MCF-7 non-invasive breast cancer cells. The antibody was able to detect endogenous FilaminA expression in all three cell lines. Quantification results revealed no significant difference in the FilaminA expression levels between these three cell lines (Figure 5.10). Having established the anti-FilaminA antibody works well in the MDA-MB-231 cell line, the interaction between PAK6 and FilaminA was explored further. MDA-MB-231 cells were transfected with full-length GFP-PAK6 and GFP-PAK6 kinase domain, proteins were isolated using GFP-TRAP beads and lysates were probed for the presence of endogenous FilaminA. Again endogenous FilaminA could be detected when full-length PAK6 was isolated but not in isolates of PAK6 kinase domain alone.

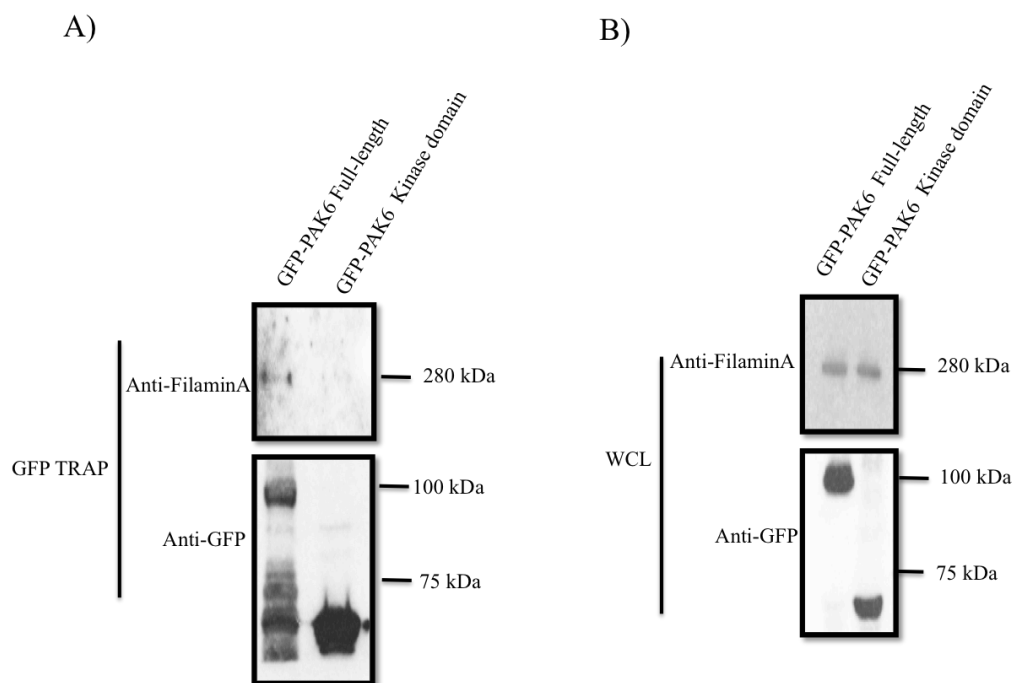


**Figure 5.9: Testing of FilaminA antibodies for detecting exogenous FilaminA.** MDA-MB-231 cells were transfected with Myc-FilaminA and detected the expression of FilaminA using anti-FilaminA from Gentex and an anti-Myc antibody. Lysates were immunoblotted for  $\beta$ -actin as a loading control.





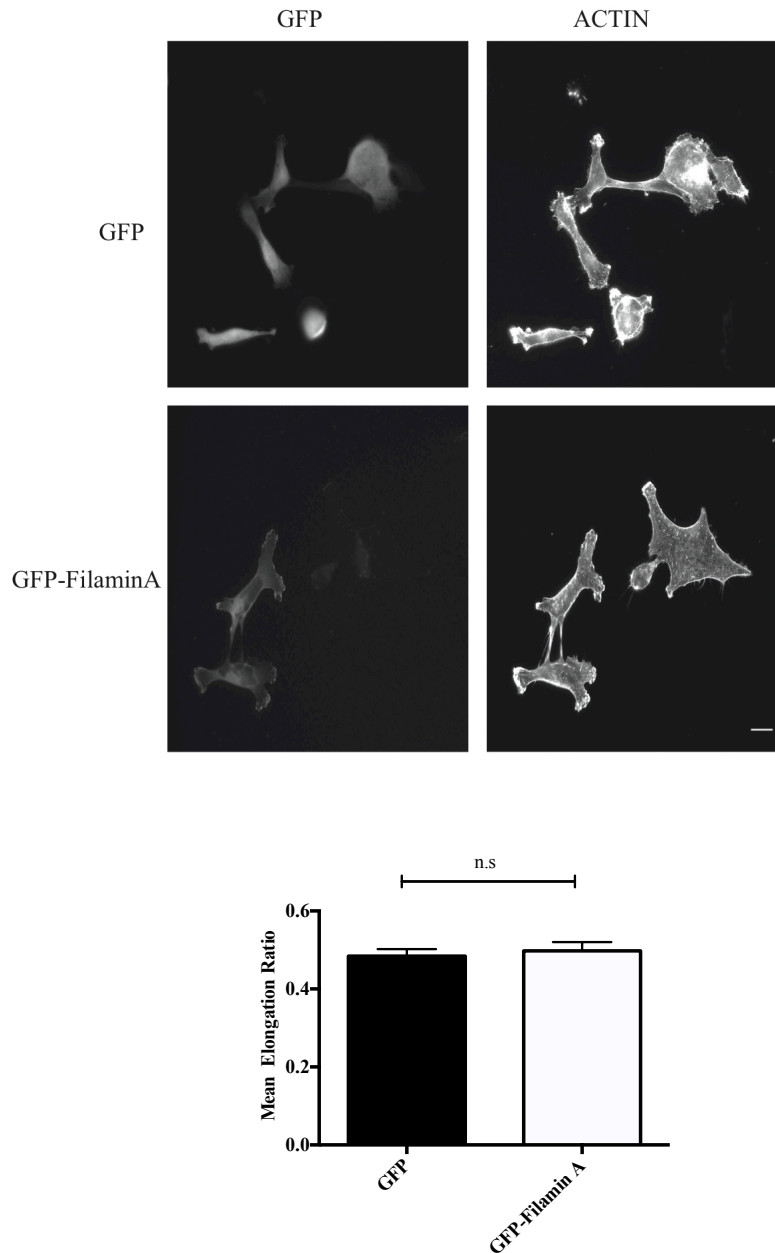
**Figure 5.10: FilaminA expression in panel of breast cell lines.** MCF-10A, MCF-7 or MDA-MB-231 whole cell lysates were immunoblotted for FilaminA expression using the anti-FilaminA antibody Gentex or the  $\beta$ -actin antibody, which was used as a loading control. Quantification results are representative of three independent experiments. Bars represent the mean  $\pm$  S.E.M.



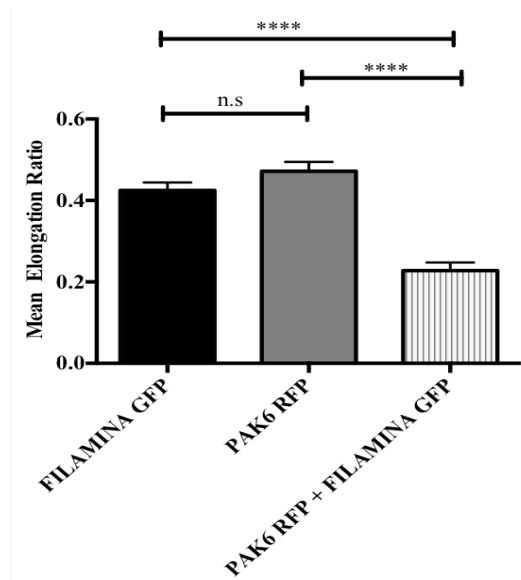
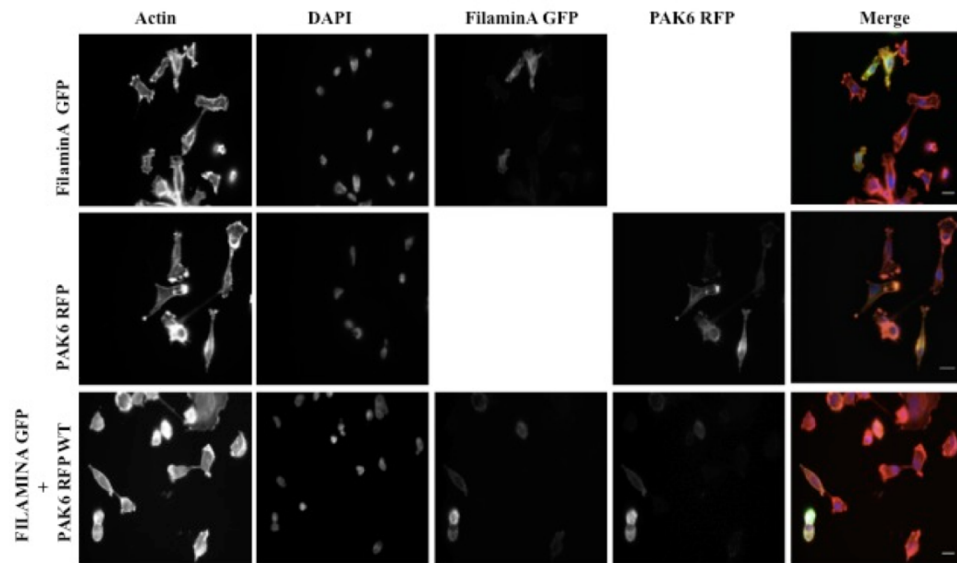
**Figure 5.11: PAK6 interacts with FilaminA in MDA-MB-231 cells.** **A)** MDA-MB-231 cells were transfected with GFP-PAK6 full-length and GFP-PAK6 kinase domain. The cells were lysed and GFP-TRAP used. The samples were immunoblotted for endogenous FilaminA using FilaminA specific antibody and GFP antibody for exogenous PAK6. **B)** MDA-MB-231 whole cell lysate (WCL) was immunoblotted for endogenous FilaminA using FilaminA specific antibody and GFP for exogenous PAK6. The blots are representative of three independent experiments.

### **5.2.7. Co-expression of FilaminA and PAK6 induces morphological changes in MDA-MB-231 cells**

In chapter 3, wild type full-length PAK6 overexpression did not induce cell morphology changes in MDA-MB-231 cells (Figure 3.18). Given that an interaction between FilaminA and PAK6 was observed in these cells (Figure 5.8), the effect of overexpressing wild type FilaminA alone or in combination with overexpressed PAK6 on cell morphology was investigated. Overexpression of GFP-FilaminA or GFP alone demonstrated that there is no difference in cell morphology in cell expressing GFP alone (control) or GFP FilaminA (Figure 5.12). In contrast, co-expression of wild type full-length PAK6 and wild type FilaminA can induce a cell morphology change in MDA-MB-231 cells (Figure 5.13) which is reminiscent of that induced by overexpressing active PAK6 alone (Figure 3.18). Indeed, overexpression of wild type full-length PAK6 alone does not induce cell rounding (Figure 3.14 and 3.18).



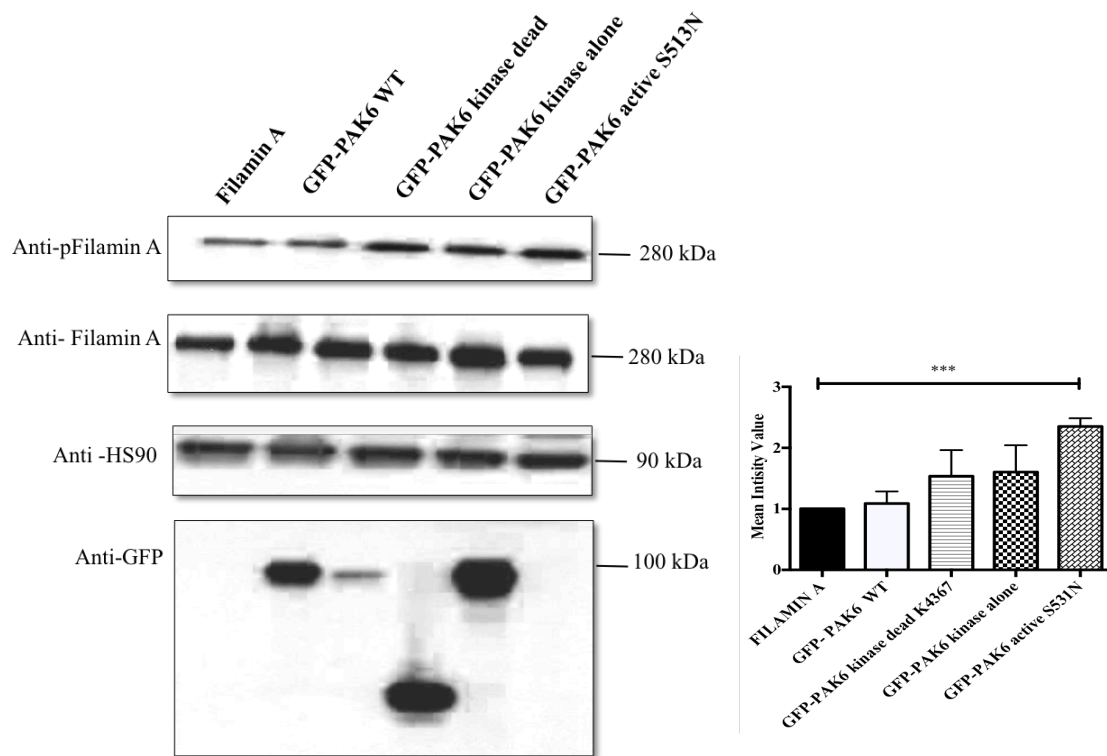
**Figure 5.12: FilaminA overexpression does not induce morphological changes in MDA-MB-231 cells.** MDA-MB-231 cells were transfected with GFP control or GFP-FilaminA alone. Cells were then fixed and stained TIRIC phalloidin for F-actin. Shape analysis was performed on the cells using Image J to determine the elongation ratio. Quantification results were performed over three independent experiments. Bars represent the mean  $\pm$  S.E.M.



**Figure 5.13: FilaminA co-expression with PAK6 induces morphological changes in MDA-MB-231 cells.** MDA-MB-231 cells were transfected with GFP control or GFP-FilaminA alone or co-transfected with wild type RFP-PAK6. Cells were then fixed and stained with TRIC phalloidin for F-actin. Shape analysis was performed on the cells using Image J to determine the elongation ratio. Quantification results perform over three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance compared with GFP control cells was calculated using Student's *t*-test; \*\*\*\*,  $P < 0.0005$ . Scale bar = 10  $\mu$ m.

### **5.2.8. FilaminA phosphorylation levels are elevated in the presence of PAK6 in MDA-MB-231 cells**

Having established that FilaminA interacts only with full-length PAK6 (Figure 5.8) but not the kinase domain of PAK6 alone, and co-expression of FilaminA and PAK6 induces cell rounding (Figure 5.13), the interaction between FilaminA and PAK6 was further explored. It is possible that FilaminA directly activates PAK6 to induce rounding or that the association between FilaminA and PAK6 scaffolds a PAK6 activator to the complex. Indeed, FilaminA is known to bind to IQGAP1 (Jacquemet et al., 2013) which in turn binds Cdc42 a known activator of PAK6. It is also possible that PAK6 could mediate regulation of FilaminA, and indeed it was reported that serine 2152 could be phosphorylated by PAK1 (Vadlamudi et al., 2002b). The phosphorylation status of the serine 2152 residue in the catalytic domain of FilaminA is thought to be an indicator of FilaminA activity (Vadlamudi et al., 2002b). Moreover, serine 2152 phosphorylation has been shown to be required for PAK1-mediated membrane ruffling and for regulation of cellular migration by Ribosomal S6 Kinase (RSK), a key kinase in the Ras-MAPK pathway (Vadlamudi et al., 2002a). The kinase domain of PAK6 did not bind to FilaminA; nevertheless PAK6 could still influence FilaminA activity indirectly. Thus, the effect of PAK6 overexpression on the phosphorylation status of endogenous FilaminA serine 2152 was tested. Cells were transfected with wildtype GFP-PAK6, kinase dead GFP-PAK6, GFP-PAK6 kinase domain alone or active GFP-PAK6 and lysates were probed with the serine 2152 phosphorylation specific antibody. Whilst overexpression of wildtype GFP-PAK6, GFP-PAK6 kinase dead, GFP-PAK6 kinase domain alone did not significantly elevate the level of FilaminA serine 2152 phosphorylation, there was a significantly different increase in the level of endogenous FilaminA serine 2152 phosphorylation when cells were overexpressing active PAK6 (Figure 5.14). This result indicates that PAK6 may influence the activity of FilaminA.



**Figure 5.14: Active PAK6 expression significantly increases FilaminA phosphorylation levels.** MDA-MB-231 cells were transfected with Myc-FilaminA, GFP-PAK6, GFP-PAK6 kinase domain, GFP-PAK6 kinase dead and GFP-Active PAK6. The cells were immunoblotted for levels of pFilaminA at serine 2152, FilaminA alone, H90 as a loading control and GFP for detect the expression of PAK6. Quantification results for the expression of pFilaminA are representative of three independent experiments. Bars represent the mean  $\pm$  S.E.M. Statistical significance calculated using Student's *t*-test; \*\*\*\*,  $P < 0.0006$ .

### 5.3. Discussion:

In this chapter, RhoV, RhoU and RhoD were identified as PAK6 binding partners. Moreover, a novel interaction between PAK6 and FilaminA has been established in MDA-MB-231 cells. Furthermore, whilst expression of PAK6 or FilaminA alone has no impact on cellular morphology, co-overexpression of wild type full-length PAK6 and FilaminA could induce cell rounding in MDA-MB-231 cells. Indeed, PAK6 indicating that might be a potential regulator of FilaminA activity.

PAKs are among the best-characterised effectors of Rac and/or Cdc42 and are considered to play a critical role in regulating actin dynamics and cell adhesion during cell migration. (Whale, 2011, Wells and Jones, 2010). However, a role for PAK6 in cytoskeletal reorganization has not been reported outside this study. Based on the findings reported in Chapter 3 and 4, PAK6 may also influence actin cytoskeletal dynamics either via interaction with Cdc42 or potentially via other GTPases. It is well established that RhoGTPases mediate the regulation of the actin cytoskeleton including the formation of filopodia (Cdc42, RhoD and RhoV) (Tapon and Hall, 1997, Gad et al., 2012, Aronheim et al., 1998, Gasman et al., 2003) or lamellipodia (Rac, RhoG) (Parri and Chiarugi, 2010, Katoh et al., 2006). Whilst RhoA can stimulate the formation actomyosin-mediated cell contraction, focal adhesion and stress fibres (Karlsson et al., 2009, Jaffe and Hall, 2005). RhoV via an interaction with PAK2 is implicated in the control of the mitogen activated protein kinase (MAP kinase), c-Jun N-terminal kinase (JNK) and reorganization of the actin cytoskeleton (Aronheim et al., 1998). More recently, it has been shown that PAK6 interacts with the atypical RhoGTPase, RhoV and RhoU (Shepelev and Korobko, 2012), which shares close sequence homology with Cdc42.

In this study, PAK6 interacts with RhoV and RhoU which is consistent with previous work (Shepelev and Korobko, 2012). However, results presented here suggest that the interaction between RhoU and PAK6 is less efficient (Figure 5.3). Additionally, the pull down work described here identified a novel interaction between PAK6 and RhoD (Figure 5.4).

PAK6 is known to bind to Cdc42 (Schantz et al., 2004) and overexpression of Cdc42 is already known to induce cell rounding (Calvo et al., 2011) thus, the impact of overexpression



of RhoV, RhoU and RhoD on cell morphology was tested in MDA-MB-231 cells. Whilst overexpression of RhoD and RhoV induced cell rounding, overexpression of RhoU did not induce cell rounding when compared to control cells (Figure 5.5). This observation may indicate that Cdc42, RhoV and /or RhoD are more likely to interact with PAK6 to induce the cell rounding phenotype. Indeed, the PAK6 interaction with RhoU was not as robust as RhoV, although overexpression of RhoU in NIH3T3 cells can reduce stress fibres formation and induces a rounded cell morphology (Saras et al., 2004).

Much less is known about the activity of RhoD in cells. It is reported that RhoD binds to a diaphanous-related formin splice variant of Dia2 known as hDia2C, which specifically binds RhoD and has been shown to play a key role in the motility of endosomal vesicles (Gasman et al., 2003). Moreover, RhoD has been found to interact with the Semaphorin receptors PlexinA1 and PlexinB1 and components of the TGF- $\beta$  signalling pathway. However, the physiological significance of these interactions has not been established (Tong et al., 2007, Barrios-Rodiles et al., 2005, Hota and Buck, 2009, Zanata et al., 2002). In addition, RhoD has been shown to induce actin stress fibres and focal adhesion dissolution and this was explained to be caused by the ability of RhoD to counteract RhoA-dependent stress a fibres formation (Murphy et al., 1996, Tsubakimoto et al., 1999). Another identified RhoD effector is FILIP1, which is a filamin interacting protein that regulates actin cytoskeletal dynamics and cell migration via FilaminA mediated pathways (Gad et al., 2012). Thus, it could be speculated that PAK6 mediated cytokeletal changes may be mediated via a RhoD/FilaminA complex. In contrast to RhoD, RhoV has been linked to junctional dynamics through interactions with the Arhgef7b( $\beta$ -Pix)/Pak complex and cadherin proteins. Moreover, RhoV is essential to stabilize the E-cadherin/ $\beta$ -catenin complex at adherens junctions (Fort et al., 2011a, Tay et al., 2010).

Furthermore, expression of RhoV induces focal adhesion assembly, lamellipodia formation and oncogenic transformation of mouse NIH3T3 fibroblasts (Aspenström et al., 2004, Chenette et al., 2005, Chenette et al., 2006). However, RhoV is not closely associated with cell rounding outside this study.

As well as Rho Family GTPases, FilaminA was also identified as an N-terminal binding protein via mass spectrometry (Figure 5.8). FilaminA is known to bind to Cdc42 (Ohta et al., 1999), as well as the RhoD binding protein FILIP1 (Gad et al., 2012), thus, this protein could provide a platform for interaction between PAK6 and RhoFamily GTPases. In addition, it is also possible that FilaminA is a downstream effector of PAK6 or is acting via IQGAP1, which also binds to Cdc42 (Gad et al., 2012, Jacquemet et al., 2013). Studies here have demonstrated that PAK6 and FilaminA co-operate to drive cell rounding, moreover expression of PAK6 leads to increased levels of FilaminA phosphorylation at serine 2512.

Filamins are a family of cytoskeletal proteins that organise actin filaments into networks and link these actin networks to cell membranes. These properties of Filamin support cell adhesion and promote signalling systems by providing a scaffold for cytoskeletal proteins and various signalling proteins (Stossel et al., 2001, Popowicz et al., 2006). It is already known that FilaminA interacts with PAK1 via the CRIB domain, which appears to be sufficient to release autoinhibition thereby stimulating PAK1 activity (Vadlamudi et al., 2002b). Conversely, FilaminA has been placed downstream of PAK1 where PAK1 phosphorylation of FilaminA at serine 2512 leads to actin cytoskeletal rearrangements (Vadlamudi et al., 2002b), thus although FilaminA did not bind to the kinase domain of PAK6, the increase of FilaminA serine 2512 phosphorylation in the presence of PAK6 could be significant in terms of the cell rounding phenotype. FilaminA also binds to IQGAP1 in a complex that acts downstream of  $\beta$ 1 integrin activation following fibronectin integrin engagement, here FilaminA and IQGAP1 suppress Rac1 activity (Jacquemet et al., 2013). This interaction could account for the cell morphology changes observed when wild type PAK6 and FilaminA are co-expressed.

In the co-expressing cells perhaps the complex is acting downstream of  $\beta$ 1 integrin activation to suppress Rac and activate the RhoA pathway, which induces cell contractility and subsequent cell rounding. Alternatively, FilaminA binds to FilGAP, that is also reported to act downstream of ROCK to inactivates Rac. Thus, again an interaction with FilaminA could bring PAK6 and RhoD together to suppress Rac and activate RhoA pathways. Indeed, it is

suggested that FilGAP is a mediator of RhoA antagonism to; suppressing leading edge protrusion and promoting cell retraction to achieve cellular polarity (Ohta et al., 2006b, Nakamura et al., 2009).

Supporting evidence for a functional interaction between PAK6 and FilaminA comes from androgen receptor studies. Both PAK6 and FilaminA directly interact with the androgen receptor (AR), regulating nuclear translocation and transcriptional action of AR in prostate cancer cells (Loy et al., 2003, Ozanne et al., 2000, Yang et al., 2001).

The interaction between PAK6 and FilaminA could also have relevance in terms of c-Met signalling. Previous work has demonstrated that PAK6 autophosphorylation is elevated downstream of HGF in prostate cancer (Fram et al., 2014), and it has recently been reported that c-Met triggered FilaminA phosphorylation at serine 2152 in HEK293 cells, or gastric cancer cells MKN-45 or GTL-16 cells, and that phosphorylation is lost upon inhibition of c-Met kinase activity. Moreover, the c-Met activation was correlated with cell rounding (Mai et al., 2014). However, in breast cancer cells no evidence of increased PAK6 autophosphorylation was detected following c-Met activation (Figure 3.6 and 3.8). Thus it will be interesting to further investigate the relationship between PAK6, FilaminA and c-Met signalling pathways.

In summary, work here has demonstrated that there is a functional relationship between PAK6 and Filamin that cooperates to induce cell rounding. PAK6 binds to FilaminA and could be a potential regulator of FilaminA activity, given that PAK6 can drive Filamin phosphorylation. Alternatively, FilaminA through its interaction with potential regulators of PAK6 activity could regulate PAK6.

## **5.4.Future work**

It will be important to establish which, if any of the Rho family GTPases influences PAK6 activity and induction of induced cell rounding. This could be investigated by reducing RhoGTPase expression in cells prior to overexpression of active PAK6. Additional experiments are required to establish if PAK6 binding to the Rho Family GTPases influences PAK6 localisation and activity.

It would be interesting to further explore the interaction between PAK6, RhoD and FilaminA, including a consideration of FILIP1. The limitations of using co-immunoprecipitation techniques to investigate protein-protein interactions do not confirm that the identified proteins pulled down together are in fact interacting directly. For example, PAK6 may be in a binary complex with other proteins. Alternative approaches to establish direct protein-protein interactions and explore these interactions further would include yeast two-hybrid screening and fluorescence resonance energy transfer (FRET).

Time constraints prevented the expansion of PAK6 studies to cell migration but future work could include an exploration of how the PAK6:FilaminA functional interaction identified here could impact on cell migration or invasion using techniques such as time-lapse microscopy and 3D spheroid assays.

## **Chapter 6:**

### **Concluding Remarks**

## **6. Chapter 6: Concluding Remarks**

The establishment of metastases in sites distant from the primary tumours is the leading cause of mortality in cancers (Valastyan and Weinberg, 2011), including breast cancer (Rahman and Mohammed, 2015). Indeed, although there are many effective treatments for breast cancer, a significant proportion of patients experience relapse and incurable metastatic disease (Higgins and Baselga, 2011). Therefore, identifying the proteins that regulate metastasis is crucial to designing therapeutic strategies that target this last stage of tumour progression. This study focused on invasive breast cancer and posed the question of whether PAK6 regulates cytoskeletal changes involved in breast cancer progression. Whilst there is already considerable evidence that PAK6 plays a role in prostate cancer progression, there is no study investigating PAK6 in breast cancer progression, with the exception of one study suggesting that there is a high level of PAK6 expression in breast cancer cell lines (Kaur et al., 2008b). This present study demonstrated that PAK6 is indeed expressed in a range of breast cancer cell lines, including cells derived from invasive breast cancer (Figure 3.3). Furthermore, using the highly invasive MDA-MB-231 cell line the study demonstrated that specifically overexpression of active PAK6 induced cell rounding (Figures 3.17 and 3.18). In direct contrast, siRNA-mediated depletion of PAK6 induced cell elongation (Figures 4.3 and 4.4). These cell morphology data were also phenocopied using pharmacological inhibition of PAK6 expression with Panobinostat (Figure 4.8). This study clearly demonstrated the important and specific role of PAK6 in mediating cell morphological changes.

The two cellular morphologies reported in this study: rounded and elongated. It is generally accepted that cancer cells have the potential to shift between an amoeboid (rounded) morphology and a mesenchymal (less-rounded) morphology during migration through matrix, and these two motility types are thought to allow cells to adapt to the environmental changes and matrix stiffness (Pankova et al., 2010).

Indeed, the amoeboid morphology is routinely linked to increased invasion potential (Gadea et al., 2008). Work presented here might suggest that PAK6 activity could promote a more rounded morphology. The rounded amoeboid mode of migration is characterised by high levels of actomyosin contractility (Wilkinson et al., 2005a) thought to be mediated through a Rho-ROCK pathway, and the more extended mesenchymal mode of migration is associated with increased levels of Rac signalling and a suppression of Rho (Friedl and Wolf, 2003, Wolf et al., 2003). This study speculates that PAK6 could be involved in the rounded morphology, thus supporting the Rho-ROCK pathway (Sahai et al., 2007, Sanz-Moreno et al., 2008, Friedl and Alexander, 2011). In support of this hypothesis, data presented here suggests that by inhibiting RhoA signalling, either directly, with the Rho-specific inhibitor C3, or indirectly by inhibiting the RhoA effector kinase, ROCK significantly impairs active PAK6 induced cell rounding and significantly induces cell elongation (Figures 4.10 and 4.11). The Rho-ROCK pathway is thought to converge on Myosin II; however, using Myosin II inhibitors did not prevent active PAK6 induced cell rounding. Therefore, how PAK6 drives contractility via Rho/ROCK remains to be elucidated.

Interestingly, ROCK is not only involved in the activation of myosin II, but is also required for activation of FilGAP, which suppresses Rac activity (Ohta et al., 2006b). Therefore, it could be speculated that an alternative pathway downstream of PAK6 and ROCK, such as FilGAP, could promote cell rounding. FilGAP is phosphorylated by ROCK, and this phosphorylation stimulates its RacGAP activity (Ohta et al., 2006a, Nakamura, 2013). Overexpression of FilGAP induces cell rounded and blebbing amoeboid morphology, which requires Rho-ROCK-dependent phosphorylation of FilGAP (Nishi et al., 2015, Saito et al., 2012), whereas depletion of endogenous FilGAP induces a Rac-driven elongated mesenchymal morphology.

It has been reported that FilGAP contributes to the regulation of the transition from mesenchymal to amoeboid (Saito et al., 2012). More importantly, it has been shown that FilGAP binds to the FilaminA, and this interaction is thought to promote GAP activity (Ohta et al., 2006a, Guilluy et al., 2011).

This study used mass spectrometry analysis of breast cancer cell lysates to identify FilaminA as a novel PAK6 binding partner (Table 5.1). Subsequently, this binding was validated using GFP-TRAP (Figure 5.8). Interestingly, whilst expression of either wild type PAK6 or wild type FilaminA alone did not induce cell rounding, co-expression of the wild type proteins led to a significant increase in rounded cells (Figure 5.13), suggesting that PAK6 and FilaminA may co-operate to induce increased cellular contractility; indeed, FilaminA may activate PAK6 as it binds in the putative N-terminal regulatory region (Figure 6.1). Phosphorylation of FilaminA at serine 2512 by PAK1 is thought to promote functionality at membrane ruffles; however, this study confirmed that FilaminA does not bind to the kinase domain of PAK6. Although, there is no interaction between FilaminA and the PAK6 kinase domain, an increase in FilaminA serine 2512 phosphorylation was observed in the presence of active PAK6. Therefore, it is important to further elucidate the role of FilaminA serine 2512 phosphorylation in the context of PAK6 activity.

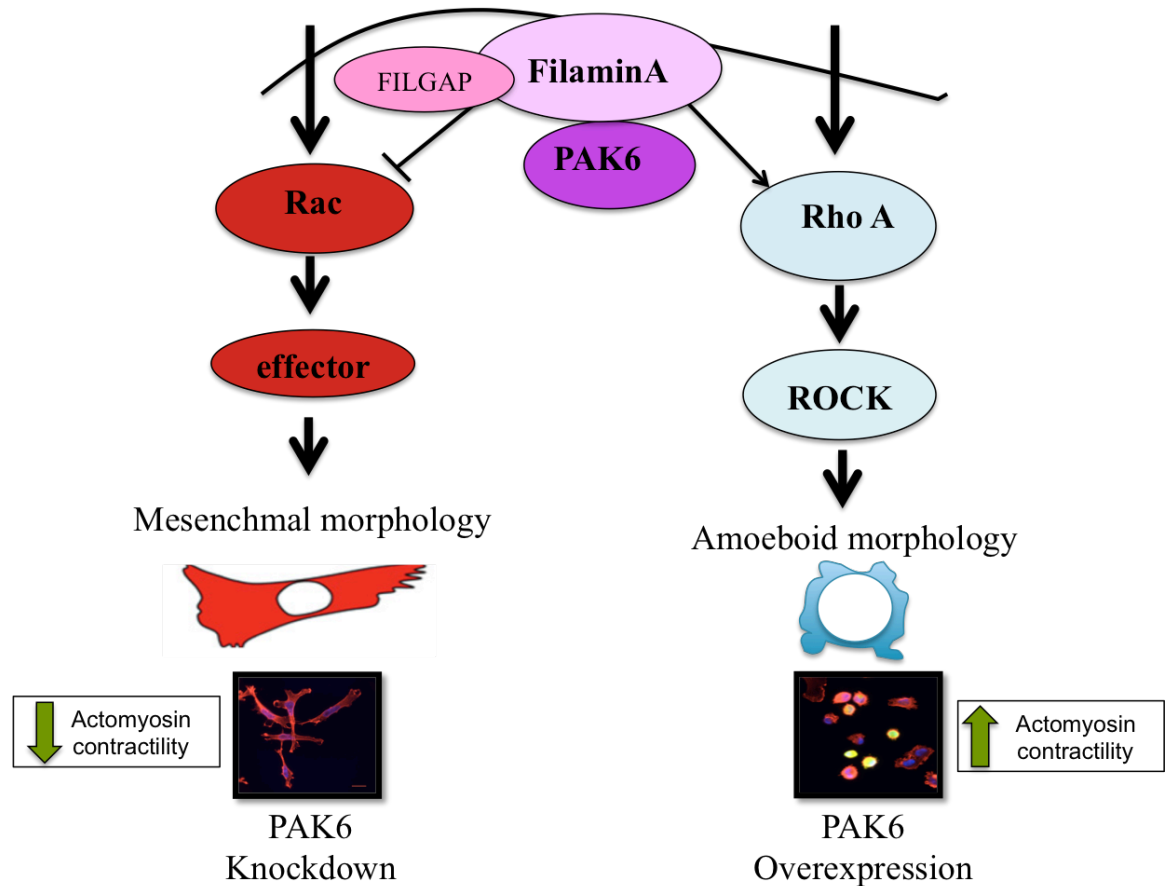
Interestingly, both PAK6 and FilaminA activity have been associated with c-Met signalling, in which overexpression of c-Met triggered FilaminA phosphorylation, which correlated with cell rounding (Mai et al., 2014). However, in breast cancer cells, no evidence of increased PAK6 autophosphorylation was detected following c-Met activation (Figure 3.7). Furthermore, using PAK6 serine 560 phosphorylation as a marker of activity is now disputed (Fram et al., 2014) and it might be prudent to revisit c-Met PAK6 signalling in breast cancer cells when better activity detecting tools are available.



Previous work has suggested that PAK family kinase activity and/or localisation may be regulated via interaction with Rho family GTPases. Prior to this study PAK6 was known to interact with Cdc42 but not Rac or RhoA (Schrantz et al., 2004). Furthermore, a single study had suggested that PAK6 might also bind to RhoU and RhoV (Shepelev et al., 2011). This study confirmed here that PAK6 could bind to RhoV, RhoU and Cdc42. In addition, this study identified a novel interaction between RhoD and PAK6. Cdc42 overexpression has been reported to induce cell rounding (Gadea et al., 2008).

Further experiments here suggest that RhoV and RhoD were also able to induce the cell rounding phenotype observed in cells overexpressing activated PAK6. At this stage, it is not possible to assign a specific PAK6 and RhoGTPase interaction with the cell rounding phenotype. However, It has been reported that RhoD, via binding to FILIP1, which is a protein that interacts with FilaminA, can control actin dynamics and, thus, cell attachment and cell migration (Gad et al., 2012). Therefore, it could be speculated that PAK6 mediated cytoskeletal changes may be mediated via a RhoD/FilaminA complex.

In conclusion, a specific role for PAK6 in mediating cell morphological changes using overexpression and knockdown strategies has been identified. Evidence has been presented to support a hypothesis whereby PAK6 can mediate increased cell contractility (cell rounding) by modulating activation of the RhoA:ROCK pathway via a novel interaction with FilaminA (Figure 6.1).



**Figure 6.1: Proposed model of PAK6 and FilaminA binding partner in regulation the change of cell morphology in breast cancer.** Schematic illustrating data gathered throughout this study provides indication that PAK6 lies downstream of RhoA and ROCK to promote cell contractility. PAK6 has been shown to bind to FilaminA, which bind to FilGAP a suppresser of Rac activity and promote cell contractility. Overexpression active PAK6 induced cell rounding. Moreover, inhibit the active PAK6 form significantly induce cell elongated upon ROCK and RhoA inhibitors. In direct contrast, siRNA-mediated depletion of PAK6 induced cell elongation, which may elevates Rac activity and promotes mesenchymal morphology.

## **Chapter 7: Appendix**

## **7. Chapter 7: Appendix**

### **7.1.Generation of PAK6 isoform specific rabbit polyclonal antibody**

PAK6 is one of the least characterised members of the PAK family. At the start of my project, a PAK6 specific antibody was commercially available from Calbiochem, and had been validated by our group (Fram et al., 2014), but unfortunately this PAK6 antibody was discontinued and no longer available. Consequently, a novel PAK6 antibody was made using human PAK6 sequences that might be exposed on the surface of the protein (Figure 7.1). This antibody is subsequently referred to as in-house PAK6 antibody.

PAK6 contains 681 amino acids and the antibody was made to recognise a region of PAK6 outside of the kinase domain. Potential PAK6 sequences were compared with the sequences of other group II PAKs and group I PAKs to identify regions in PAK6 that are unique. Protscale software (available on the Expasy website at <http://web.expasy.org/protscale>) was used to identify the suitable peptide sequence on PAK6 for antigen recognition. Two different scales (Hopp & Woods, and Kyte & Doolittle) were used to assess the hydrophobicity and the hydrophilicity profiles of PAK6 sequences (Figure 7.1). The most ideal antigenic epitopes are hydrophilic, as these regions tend to be found on the surface of proteins and are available for antibody recognition, whereas hydrophobic regions maybe hidden in the protein interior.

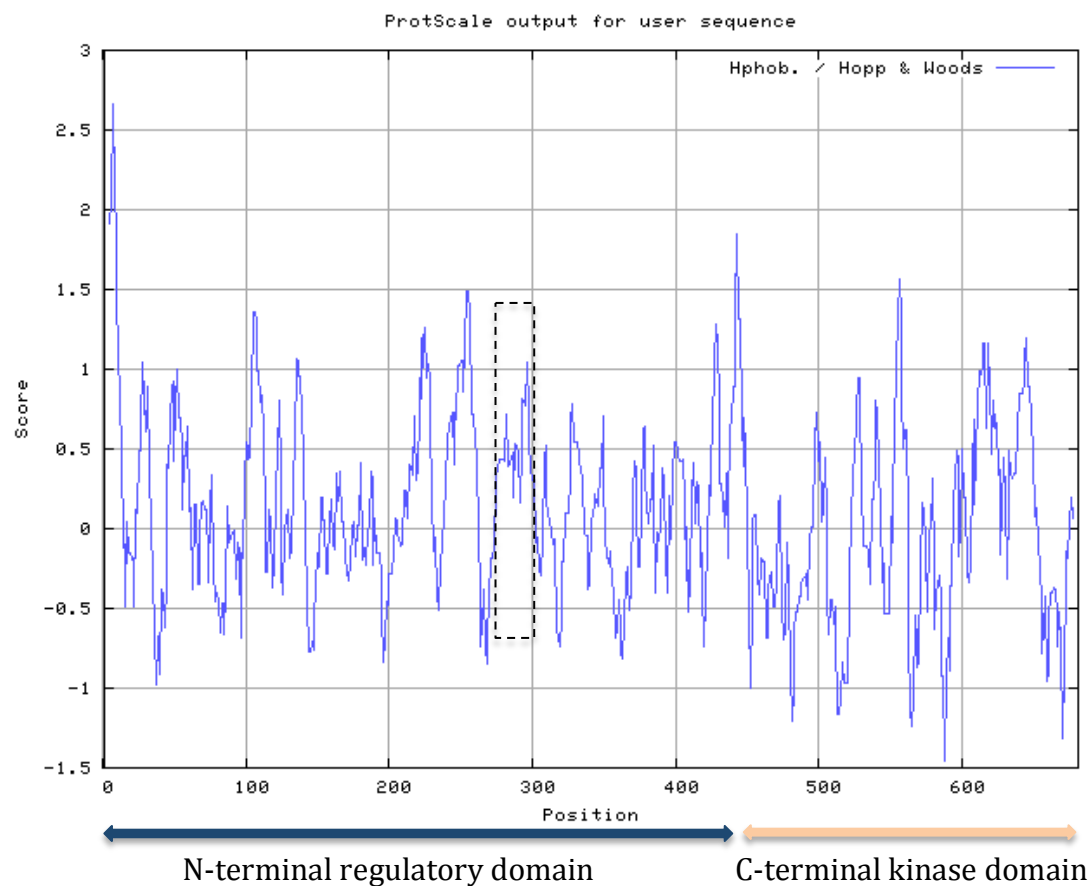
Subsequently, amino acids sequences between 181-350 are the most suitable and are unique to PAK6 (Table 7.1). The ideal length of immunizing peptide sequence is 10-20 amino acids for antibody preparation (Hancock and O'Reilly, 2005). This reflects the fact that sequences above 20 amino acids in length might lose specificity and induce a secondary reaction, conversely if the peptide is too short, it could be non-specific and not recognise the protein with sufficient affinity. Four putative peptides were sent to the Custom peptide antibody production services at Eurogentec (Table 7.1). They recommended GRPGGEGSPSPKTRE

and QSKPNSSFRPPQKD. The peptide sequence QSKPNSSFRPPQKD was preferred to correspond and this chosen sequence to amino acid 284-298 of the PAK6 protein, located on the N-terminal regulatory domain. The Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/>) was used to check for all PAK and other proteins, which contain similar peptide sequence, and may also interact with the antibody designed. The search using protein BLAST indicated that this peptide sequence was specific for PAK6. The peptide identified was manufactured and inoculated into rabbit hosts for polyclonal antibody production by Eurogentec Ltd.

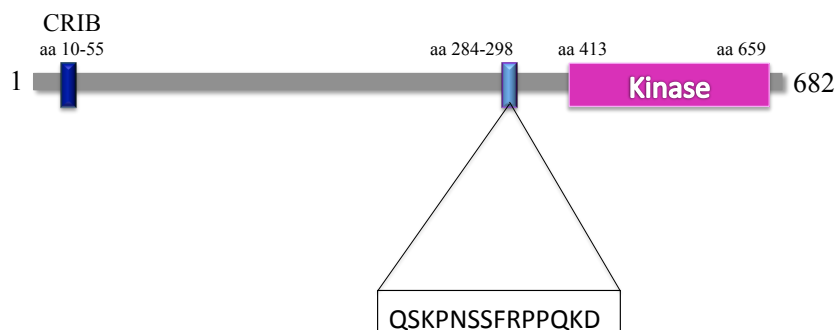
**Table 7.1:** The sequences of PAK6. Four sequences between 181-350 are the most suitable and are unique to PAK6 using two different scales (Hopp & Woods, and Kyte & Doolittle) to assess the hydrophobicity and the hydrophilicity profiles of PAK6 sequences.

Sequences
195-215: LGACLQSSPPGASPPTGTNR
225-245: EEARPQSCLVGSATGRPGGE
258-278: RRLFRSMFLSTAATAPPSSSK
300-320: SLVAKAQSLPSDQPVGTFSP

A)



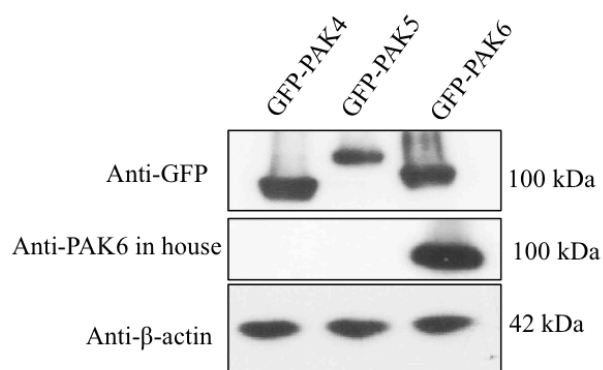
B)



**Figure 7.1: Generation of the in-house PAK6 antibody.** A) Hopp and Woods hydrophobicity/hydrophilicity profile of PAK6 protein was used for epitope selection (as indicated it in the box). B) Epitope used for antibody presentation (as indicated in the box) within the N-terminal regulatory domain of PAK6.

## **7.2.Validation of the in-house PAK6 antibody**

Previous studies have shown that endogenous PAK6 is expressed in DU145, HT29 and MDA-MB-231 cells (Fram et al., 2014, Kaur et al., 2008b). To validate the specificity of in-house PAK6 antibody, lysates from cells overexpressing GFP-PAK4, GFP-PAK5 and GFP-PAK6 were tested. The in-house PAK6 detected GFP-PAK6, but not GFP-PAK4 or GFP-PAK5 (Figure 7.2). The whole cell lysates were also probed with a GFP-specific antibody to confirm that all three PAK proteins were expressed (Figure 7.2). The ability of the in-house PAK6 antibody to detect endogenous PAK6 was also tested, and western blotting experiments showed that the antibody was not able to detect an endogenous protein band at correct size. The PAK6 in-house antibody was therefore able to specifically detect transfected PAK6, but did not appear to detect endogenous PAK6 expression of the correct apparent size. During antibody development, a new commercial anti-PAK6 antibody became available and could detect PAK6 in MDA-MB-231 cells at the expected size, as illustrated in Figure 3.1.



**Figure 7.2: Validation the PAK6 in-house antibody.** Testing the in-house PAK6 antibody. HEK293 cells overexpressing GFP-PAK6, GFP PAK5 and GFP-PAK4 were lysed and immunoblotted for PAK6 expression using a PAK6 in-house antibody. PAK6 in-house antibody detects GFP-PAK6 but not GFP-PAK4 or GFP-PAK5. Lysates were also immunoblotted for  $\beta$ -actin as a loading control. The blot shown is representative of three independent experiments.



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